

MODULAÇÃO DIFERENCIAL DE EXOPOLIFOSFATASES NO  
METABOLISMO NUCLEAR E MITOCONDRIAL DURANTE A  
EMBRIOGÊNESE DO CARRAPATO *Rhipicephalus (Boophilus)*

*microplus*

**ELDO CAMPOS**

UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE – DARCY  
RIBEIRO – UENF  
CAMPOS DOS GOYTACAZES - RJ  
JULHO – 2008

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“Tese apresentada ao Centro de Biociências e Biotecnologia da Universidade Estadual do Norte Fluminense, como parte das exigências para obtenção do título de Doutor em Biociências”.

ORIENTADOR: Prof. Dr. Carlos Logullo

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Aprovada em 25 de Julho de 2008.

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“A coisa mais bela que o homem pode experimentar é o mistério. É essa emoção fundamental que está na raiz de toda ciência e toda arte.”  
(Albert Einstein).

## **Agradecimentos:**

Aos meus pais, porque sem eles o meu caminho seria muito mais tortuoso.

Meu agradecimento especial ao Prof. Carlos Logullo pelo acolhimento em seu laboratório, por acreditar na minha capacidade e, principalmente, por sua amizade durante a realização deste trabalho. Sou-lhe grato pela enorme contribuição para o meu crescimento acadêmico-científico. Obrigado.

Ao Prof. Arnoldo Façanha pelas críticas, sugestões e amizade durante o desenvolvimento desta tese. Muito obrigado por contribuir com a minha formação acadêmico-científica. Sou muito grato por poder subir nos ombros de dois gigantes.

A Prof<sup>a</sup>. Denise Valle, por ter contribuído com a minha formação durante a revisão do artigo de 2006.

Ao Prof. Itabajara Vaz pela orientação durante o meu doutorado-sanduíche e pela contribuição científica e amizade ao longo destes anos de colaboração.

A Prof<sup>a</sup>. Aoi Masuda por ter me acolhido por 4 meses em seu laboratório durante o meu doutorado-sanduíche.

Ao Prof. Jorge Moraes, um grande amigo que me ajudou muito desde o início da minha iniciação científica.

A minha noiva, Aline, que sempre me ajudou a tomar decisões e, mais que isso, me apoiou e incentivou. Teve muita paciência (às vezes nem tanta) e entendeu (ou pelo menos tentou entender) o porquê de tantas horas no laboratório.

Agradeço a todos os meus professores, amigos e colegas que, de alguma forma, contribuíram para o desenvolvimento desta tese.

Sou muito grato também a CAPES, CNPq e FAPERJ pelos financiamentos durante toda a minha formação, e à Universidade Estadual do Norte Fluminense – Darcy Ribeiro, pelo ensino gratuito e de qualidade.

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## **Resumo**

O metabolismo de polifosfato foi caracterizado durante a embriogênese do *Rhipicephalus (Boophilus) microplus*, por meio de análises do conteúdo de polifosfato e da atividade exopolifosfatásica ensaiada nas respectivas frações subcelulares. Durante a embriogênese, o conteúdo de polifosfato se manteve constante até o 7º dia de desenvolvimento, decaindo rapidamente neste estágio caracterizado pela segmentação do embrião. Este processo mostrou relação com o decréscimo de tamanho da cadeia dos polifosfatos e com a atividade da exopolifosfatase total. Todavia, tal atividade não refletiu o conteúdo de ortofosfato, o qual se elevou somente no final da embriogênese. Após a celularização do embrião, o conteúdo endógeno de polifosfato nuclear diminuiu linearmente até o final da embriogênese, em paralelo com o aumento da atividade exopolifosfatásica. Por outro lado, a utilização do polifosfato mitocondrial ocorreu entre a celularização e a segmentação do embrião, ou seja, do 5º ao 7º dia de desenvolvimento. A atividade exopolifosfatásica nuclear foi estimulada cerca de duas vezes por RNA total, enquanto a atividade mitocondrial foi insensível a este polinucleotídeo. Somente a atividade exopolifosfatásica mitocondrial foi estimulada por concentrações fisiológicas de NADH e ADP, e completamente inibida por P<sub>i</sub>. Esta atividade também aumentou na presença de substratos respiratórios, ácidos pirúvico e succínico, e este efeito desapareceu com a adição de cianeto de potássio. A respiração mitocondrial foi ativada por ADP usando polifosfato como única fonte de P<sub>i</sub> e esta ativação foi inibida por heparina, um inibidor de exopolifosfatas. Após o isolamento da fração solúvel e de membrana mitocondrial, foi detectada atividade exopolifosfatásica nas frações solúvel e de membrana. O K<sub>map</sub> utilizando poly P<sub>3</sub>, poly P<sub>15</sub> e poly P<sub>65</sub> como substratos foram quase os mesmos para a fração nuclear, enquanto a mitocondrial apresentou uma afinidade 10 vezes maior para poly P<sub>3</sub> comparando com o poly P<sub>15</sub> e poly P<sub>65</sub>. A atividade exopolifosfatásica foi estimulada duas vezes por Mg<sup>+2</sup> e Co<sup>+2</sup> na fração nuclear e somente por Mg<sup>+2</sup> na fração mitocondrial. A heparina inibiu ambas as atividades das exopolifosfatas em até 95%, porém a enzima mitocondrial foi mais sensível, apresentando um IC<sub>50</sub> de 0,2 µg/mL enquanto a nuclear teve IC<sub>50</sub> de 0,8 µg/mL. Estes resultados são consistentes com a existência de pelo menos duas diferentes isoformas de exopolifosfatas operando no núcleo e

na mitocôndria, sendo ambas moduladas de forma diferencial em função do metabolismo de cada organela durante a embriogênese do carrapato *R. microplus*.

## **Abstract**

The polyphosphate metabolism was characterized during *Rhipicephalus (Boophilus) microplus* embryogenesis by polyphosphate content and exopolyphosphatase activity in respectively subcellular fractions. The decline in total polyphosphate content after the 7<sup>th</sup> day of embryogenesis does not reflect the free phosphate ( $P_i$ ) increase and the total polyphosphate chain length decrease after embryo cellularization, reflecting the exopolyphosphatase activity. The endogenous nuclear polyphosphate decrease and exopolyphosphatase activity increased after embryo cellularization until the end of embryogenesis, while the utilization of mitochondrial polyphosphate content occurred between the embryo cellularization and segmentation stages, days 5 to 7<sup>th</sup> of development. The nuclear exopolyphosphatase activity was stimulated about two times by total RNA, but no effect was observed on mitochondrial exopolyphosphatase. Only the mitochondrial exopolyphosphatase activity was stimulated by physiologic concentrations of NADH and ADP, and completely inhibited by  $P_i$ . This activity also increased in the presence of the respiratory substrates pyruvic and succinic acids and this stimulatory effect disappeared upon addition of potassium cyanide. Mitochondrial respiration was activated by ADP using polyphosphate as the only source of  $P_i$  and this activation was inhibited by heparin, an exopolyphosphatase inhibitor. The exopolyphosphatase activity was also analyzed after mitochondrial soluble and membrane fractions isolation and the activity was detected in either fraction, membranar and soluble. The  $K_{mapp}$  utilizing poly  $P_3$ , poly  $P_{15}$  and poly  $P_{65}$  as substrate was almost the same for the nuclear fraction, while mitochondrial fraction showed an affinity 10 times higher for poly  $P_3$  than for poly  $P_{15}$  and poly  $P_{65}$ . The exopolyphosphatase activity was stimulated around two times by  $Mg^{2+}$  and  $Co^{2+}$  in the nuclear fraction and only by  $Mg^{2+}$  in the mitochondrial fraction. Heparin inhibited both exopolyphosphatase activities until 95%, although the mitochondrial fraction was more sensitive with an  $IC_{50}$  0,2  $\mu g / mL$ , while the nuclear fraction had an  $IC_{50}$  0,8  $\mu g / mL$ . Altogether, these data are consistent with the existence of at least two different isoforms of exopolyphosphatase operating in the nuclei and mitochondria, and both are modulated specifically by the metabolism of each organelle during embryogenesis of the hard tick *R. microplus*.

## **INTRODUÇÃO**

Eldo Campos, 2008

## 1 Introdução

### 1.1 O carapato *Rhipicephalus (Boophilus) microplus* e sua importância econômica

Os carapatos são artrópodes ectoparasitas hematófagos subdivididos em duas grandes famílias, *Argasidae* e *Ixodidae* (Sonenshine et al., 2006). São encontrados em quase todas as regiões do mundo, com predominância em áreas tropicais e subtropicais, abrangendo regiões com produção de gado de corte e leiteiro na América, África, Ásia e Austrália (Johnston et al., 1986). No Brasil, eles são descritos em aproximadamente 96% dos municípios (Horn, 1983). Possuem a capacidade de infestar vertebrados terrestres, tais como: mamíferos, pássaros, répteis e até anfíbios (Sonenshine et al., 2006). São capazes de transmitir várias doenças aos seres humanos e aos animais, e nenhum outro artrópode conhecido tem a capacidade de ser vetor de uma tão ampla variedade de organismos patogênicos. Transmitem bactérias, vírus, fungos, riquétsias e protozoários, que resultam frequentemente em infecções letais, e podem, ainda, induzir algumas toxemias através de suas picadas nos hospedeiros (Sonenshine et al., 2006).

Os argasídeos, carapatos moles, alimentam-se do sangue dos seus hospedeiros repetidas vezes, abandonando-os após cada alimentação (Sonenshine et al., 2006). As fêmeas efetuam várias posturas, alternando com a alimentação sangüínea. Cada postura não ultrapassa 150 ovos, um número pequeno quando comparado aos cerca de 3000 ovos dos ixodídeos, carapatos duros. Nos ixodídeos a alimentação é prolongada, ingerindo grandes quantidades de sangue, chegando a atingir cem vezes a sua massa corporal inicial. O sangue é utilizado como única fonte de energia para o desenvolvimento destes carapatos (Sonenshine et al., 2006).

O *R. microplus* (Figura 1), o principal representante da família dos ixodídeos, é originário da Ásia e o seu principal hospedeiro é o bovino, embora seja capaz de completar, eventualmente, o seu ciclo em búfalos, ovelhas, cavalos e veados (Sonenshine, 1991).

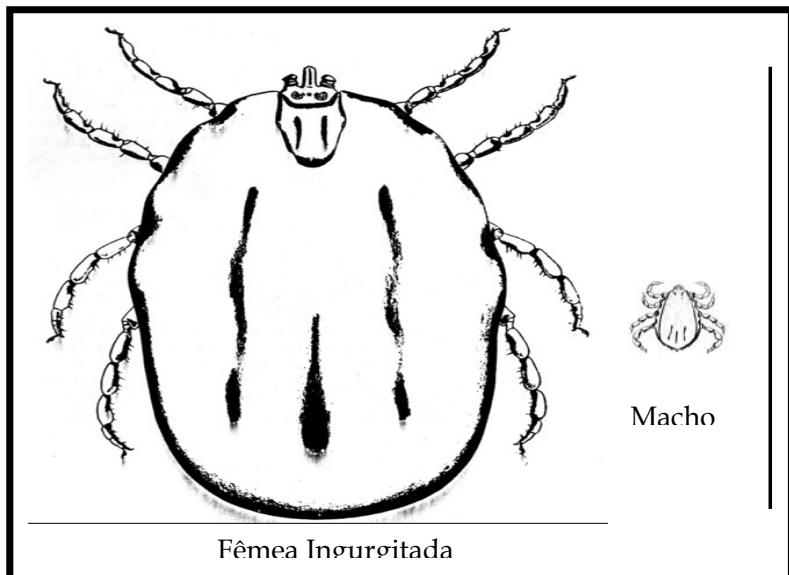


**Figura 1:** O carapato *R. microplus* parasitando seu hospedeiro, o boi (*Bos taurus*).  
Retirado de [www.icb.usp.br/~marcelcp/Boophilus.htm](http://www.icb.usp.br/~marcelcp/Boophilus.htm).

É um carapato que está amplamente distribuído na América, África, Ásia e Oceania, entre os paralelos 32°N e 32°S (Johnston et al., 1986), sendo um dos principais parasitos que afetam a pecuária destas áreas. Assim como todos os ixodídeos, ele possui como características diagnósticas uma placa esclerotizada na superfície do corpo, denominado de escudo quitinoso, que serve de sustentação a importantes músculos do corpo, quatro pares de patas, corpo composto de céfalo-torax e abdômen e peças bucais modificadas (Sonenshine, 1991). Diferentemente de outras espécies de ixodídeos, o gênero *Rhipicephalus* possui olhos, e seus palpos são extremamente curtos, inseridos dorsalmente e lateralmente. Uma característica comportamental do gênero é o parasitismo monoxeno, tendo apenas um hospedeiro em todo o seu ciclo de vida (Sonenshine, 1991).

O *R. microplus* acarreta diversos danos econômicos (Horn e Arteche, 1985), sendo considerado o principal ectoparasito dos rebanhos de gado bovino (George, 2000). Durante o repasto sanguíneo a fêmea obtém uma massa que pode atingir até 10 vezes o tamanho macho, tornando-se ingurgitada (Figura 2). Segundo Guerrero et al. (2006), este parasita causa perdas anuais de centenas de milhões de dólares no mundo todo. Além da espoliação ao couro, causada por reações inflamatórias nos locais de fixação do carapato (Seifert et al., 1968), existe ainda uma grande perda na produção de leite e carne (Sutherst, 1983) devido à perda de sangue do animal que pode atingir 2 a 3 mL de sangue/carrapato (Sonenshine et al., 2006). Ainda, *R.*

*microplus* é um importante vetor de doenças, como a tristeza parasitária bovina, causada por protozoários do gênero *Babesia* e pela riquetsia do gênero *Anaplasma* (McCosker, 1981; Young e Morzaria, 1986). Além das perdas relacionadas à bovinocultura em si, existem diversos prejuízos relacionados à mão-de-obra necessária para o controle desse parasito, despesas com instalações, compra de acaricidas e equipamentos adequados para sua aplicação, entre outros (Jamroz et al., 2000). Sendo o Brasil hoje um dos maiores produtores de carne bovina do mundo, com um rebanho bovino de aproximadamente 200 milhões de cabeças, e uma produção em torno de 8,5 milhões de toneladas de carne e 23 bilhões de litros de leite por ano (IBGE, 2006; MAPA, 2006), os custos com o controle químico podem chegar a uma ordem de 2 bilhões de dólares, além de representar um risco à saúde humana, através de resíduos na carne e no leite, e ao meio ambiente, através da contaminação da água e dos solos (Fernandes et al., 2006).



**Figura 2: Carapato bovino *R. microplus*. Fêmea ingurgitada (esquerda) e macho (direita). Retirado de Flechimann (1976).**

Tendo em vista a problemática do uso de pesticidas, a possibilidade de desenvolver uma vacina contra *R. microplus* para proteger o bovino por indução de uma resposta imune tem sido testada por diferentes grupos de pesquisa há

quase três décadas (McGowan et al., 1980; Willadsen e Kemp, 1988; da Silva, Jr. et al., 1998; Willadsen, 2001). A resistência adquirida mediada imunologicamente (Allen, 1989) pode ser aferida pela redução no número de carapatos que se fixam ao hospedeiro, pela diminuição no peso das teleóginas, e pela redução da produção de ovos e, consequentemente, de larvas (Wikle e Bergman, 1997). Estes parâmetros estudados fornecem bases para as futuras tentativas de utilização de vacinas no controle de ectoparasitas.

Para a produção de uma vacina comercial contra qualquer espécie de carapato é necessário antes de tudo identificar抗ígenos protetores; produzir, então, essas proteínas como抗ígenos recombinantes, de forma a tornar a produção economicamente viável; e formular uma vacina com抗ígenos capazes de estimular uma resposta imunológica efetiva contra o ectoparasita (Willadsen, 2001). Embora as vacinas contra carapato hoje não tenham em princípio o mesmo efeito imediato dos produtos químicos usados atualmente e não protejam totalmente o animal, não se corre com elas o risco de contaminação dos alimentos e do ambiente por resíduos químicos, e podem ainda ser espécie-específicas (Pruett, 1999).

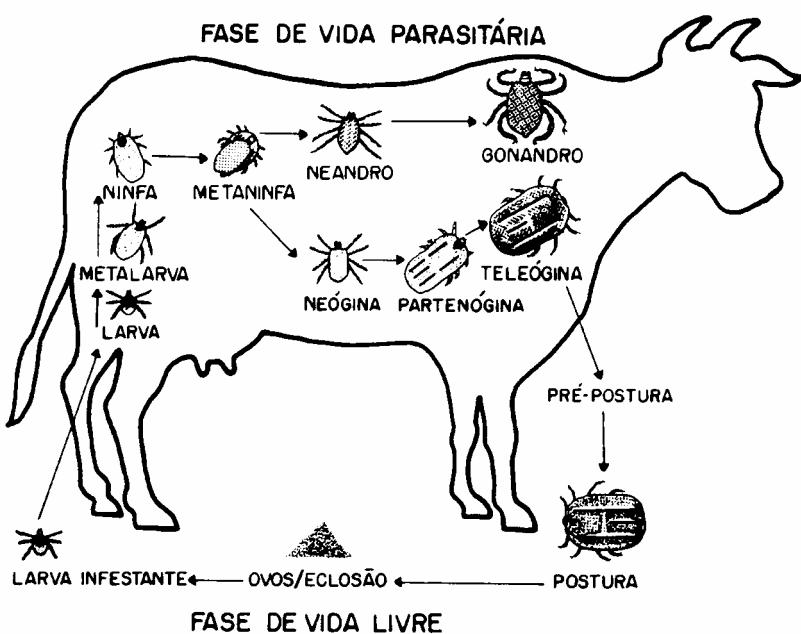
Uma alternativa para evitar o emprego de compostos químicos no controle do carapato, além do controle imunológico, é o controle biológico. Este tipo de estratégia pode ser utilizado de diversas formas: baseado nas relações ecológicas entre o carapato e o meio onde ele se encontra e entre ele e seus predadores naturais, e na utilização de compostos naturais obtidos para utilização de seu controle.

O carapato, como qualquer organismo, tem sua viabilidade relacionada a condições de estresse ambiental a que estiver submetido; assim sendo, as condições climáticas, como temperatura e umidade no campo, desempenham papel importante no equilíbrio das populações deste parasita, diminuindo ou aumentando os índices de infestação nos bovinos (Gonzales, 1995). A vegetação também pode atuar como um fator limitante no crescimento da população de carapatos. Algumas pastagens podem dificultar a sobrevivência das larvas, diminuindo a população de carapatos por sua ação repelente ou tóxica, como é o caso das plantas do gênero *Stylosanthes* (Sutherst et al., 1982) ou ainda por imobilizarem as larvas através de suas secreções ou estruturas da planta, como ocorre com o capim gordura (*Melinis minutiflora*)

(Farias et al., 1986). Uma forma alternativa de controle seria o sistema de rotação de pastagens, no qual uma área fica livre de rebanho por um determinado período de tempo, de forma a impedir a sobrevivência das larvas. No entanto, esta prática não pode ser aplicada em uma propriedade rural de pequeno porte por limitações de espaço físico (Farias et al., 1986).

## 1.2 O ciclo de vida do *Rhipicephalus (Boophilus) microplus*

Este carapato apresenta duas etapas distintas no seu ciclo de vida (Figura 3): uma fase parasitária, durante um período médio de 22 dias sobre um único hospedeiro, e uma fase de vida livre, que ocorre no solo, podendo durar de dois a três meses, dependendo fundamentalmente das condições climáticas existentes (Gonzales et al., 1974).



**Figura 3:** Representação esquemática do ciclo de vida do carapato *R. microplus*. Retirado de Gonzales et al. (1974).

Na fase parasitária, o carapato apresenta três variações morfológicas distintas: larva, ninfa e adulto. A larva apresenta três pares de patas, é bastante ativa, pois necessita encontrar o hospedeiro para nele se fixar e sobrevive às expensas das reservas de alimento acumuladas na fase de ovo. Fixa-se em locais específicos do hospedeiro utilizando algumas importantes estruturas,

como as quelíceras, as quais seccionam a pele para a introdução do hipostômio, órgão responsável pela fixação da larva na pele do bovino (Gonzales et al., 1974).

Após a fixação, a larva alimenta-se e inicia o processo de desenvolvimento e crescimento tegumentário. Passa por um período de inércia entre o 4º e 5º dia e atinge a fase de metalarva. Em torno do 6º dia, adquire uma nova estrutura, com outro tegumento, mais um par de patas e uma fileira de dentição do hipostômio entre outras alterações: é a fase de ninfa. Esta fase dura em média dois a quatro dias, sendo que ao continuar seu desenvolvimento, uma nova alteração no exoesqueleto se processa, havendo um período igual de inatividade, denominado de metaninfa, para que ao final do processo surja o indivíduo adulto, sexualmente diferenciado. Isto acontece em torno do 12º dia (Gonzales et al., 1974).

A partir dessa fase, inicia-se o processo de maturação dos machos e das fêmeas, sendo que em torno do 17º dia os machos já estão aptos à cópula. Nota-se um crescimento mais acentuado do tegumento nessa fase, sendo que nas últimas horas próximas ao ingurgitamento completo, a alimentação intensifica-se, a ponto das fêmeas apresentarem um tamanho cerca de 10 vezes superior ao dos machos. No entanto, aos 22 dias, a maioria das fêmeas cai ao solo. Os machos podem permanecer no bovino por mais de 38 dias fecundando inúmeras fêmeas. Após serem fecundadas, as fêmeas passam de metaninfa para neógena num período médio de 17 dias. Em seguida, em um período de três dias, passam a partenógena (parcialmente ingurgitada) e em mais dois dias, à teleógena (ingurgitamento máximo) (Gonzales et al., 1974).

A fase não parasitária compreende os estágios de fêmea adulta (teleógena), ovo e larva infestante. A fêmea adulta fecundada, ao desprender-se do bovino procura um local no solo para efetuar a postura. Em condições adequadas de temperatura (26-27º C) e umidade (~80%) a postura pode ser iniciada a partir do terceiro dia após a queda, podendo se estender até 60 dias no meio ambiente. Após a postura, a fêmea apresenta uma coloração mais amarelada chegando à morte após o término da ovoposição. Os ovos podem iniciar a eclosão a partir da quarta semana após o início da postura. No meio ambiente, este processo pode ser longo, sendo uma forma estratégica de sobrevivência do parasita frente às adversidades climáticas. As larvas

necessitam de um período de maturação médio de uma semana para estarem aptas a fixarem-se no hospedeiro e continuarem o desenvolvimento. Após esse período, deslocam-se às extremidades da vegetação para alcançarem mais facilmente o bovino. Nessa fase de larva infestante, elas podem sobreviver por até 36 semanas (Gonzales et al., 1974).

### **1.3 Ovogênese e Embriogênese**

Durante a ovogênese são armazenadas grandes quantidades de proteínas, lipídeos e açúcares para o crescimento dos ovócitos. A principal proteína de reserva dos ovos de artrópodes é a vitelina, a qual é derivada de um precursor hemolinfático, a vitelogenina. A vitelogenina é adquirida pelos ovócitos através de endocitose mediada por receptor (Sappington e Raikhel, 1998) e é acumulada em estruturas chamadas grânulos de vitelo. Uma das funções da vitelina é suprir o desenvolvimento do embrião com aminoácidos, e sua utilização está relacionada à ação de proteases (Fagotto, 1990; Yamamoto e Takahashi, 1993; Logullo et al., 1998).

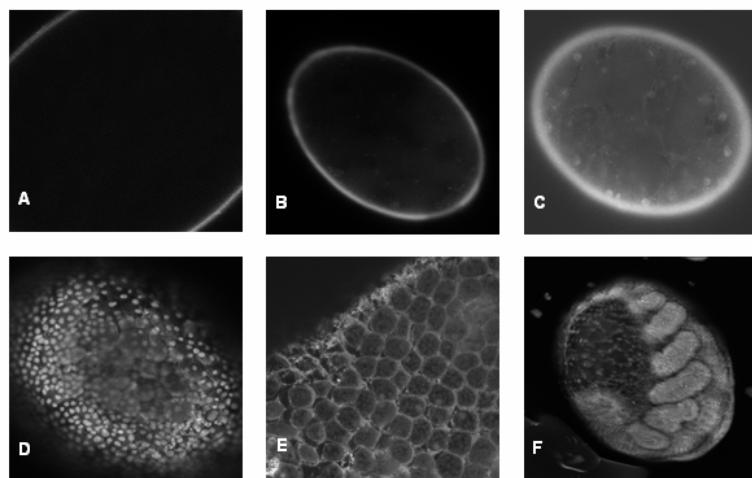
Vitelinas de todos os grupos de artrópodes são lipoglicoproteínas fosforiladas de alto peso molecular. No entanto, a mais peculiar característica das vitelinas de carrapatos é a presença de heme associada à proteína, dando aos ovos a sua cor marrom escuro (Boctor e Kamel, 1976; Rosell e Coons, 1991). O *R. microplus* obtém seu heme da hemoglobina presente no sangue ingerido (Logullo et al., 2002), sendo descrito como o primeiro organismo multicelular conhecido que não é capaz de sintetizar o heme (Braz et al., 1999). Um importante resultado dessa descoberta no contexto da reprodução de carrapatos é que os ovos devem conter todo o heme necessário para a construção de um novo organismo. Isso explicaria o motivo das vitelinas de carrapatos serem heme proteínas. Assim, além de prover aminoácidos para o embrião, a vitelina é uma reserva estratégica de heme usada para o crescimento desse artrópode (Logullo et al., 2002).

Diferentemente de aves e mamíferos que apresentam durante o seu desenvolvimento várias divisões mitóticas formando estruturas como a mórula (16 células), o *R. microplus* se desenvolve inicialmente formando um sincício acelular com abrupta celularização, como é observado na mosca *D.*

*melanogaster* (Bate e Arias, 1991). Porém, diferentemente desta, em carapatos este evento ocorre em um tempo muito longo, levando em média 20 dias desde a postura dos ovos até a eclosão (Sonenshine, 1991).

Os principais eventos em *D. melanogaster* são: fertilização, fusão dos núcleos do espermatozóide e do óvulo gerando o zigoto que passa por rápidas divisões mitóticas, uma a cada nove minutos. Em seguida, o processo é realizado sem clivagem do citoplasma, resultando em uma estrutura denominada sincício, na qual muitos núcleos estão presentes em um único citoplasma. Até esse momento, o embrião é considerado unicelular. Após nove divisões, os núcleos migram para a periferia formando o blastoderma sincicial. Posteriormente, membranas crescem a partir da superfície envolvendo os núcleos e formando células, que dão origem ao blastoderma celular. No entanto, não são todos os núcleos que dão origem a esta estrutura. Aproximadamente 15 núcleos se posicionam na extremidade posterior do embrião, desenvolvendo-se em células polares, que futuramente darão origem às células germinativas, ou seja, espermatozoides ou óvulos (Bate e Arias, 1991; Monnerat et al., 2002).

Recentemente, alguns momentos morfológicos mais marcantes na embriogênese do *R. microplus* foram descritos pelo nosso grupo (Campos et al., 2006). Conseguiu-se identificar a formação de um sincício acelular no terceiro dia (Figura 4C), do blastoderma celular no quinto dia (Figura 4D) e a completa segmentação do embrião no sétimo dia do desenvolvimento (Figura 4F).



**Figura 4:** Embriogênese do *R. microplus*. Ovos permeabilizados de diferentes dias após a ovoposição foram submetidos a microscopia confocal de varredura a laser, somente com auto fluorescência (A) e com os marcadores Laranja de acridina (B, C e D) e Azul de Evans (E). Embriões no sexto (A, D e E), primeiro (B), quarto (C) e sétimo (F) dia de desenvolvimento. Note que somente o córion é autofluorescente (A). Laranja de acridina e Azul de Evans mostram o núcleo e o limite das células respectivamente. Projeção da reconstrução em 3D do embrião é mostrada em F. Retirado de Campos et al. (2006) em Anexo I.

#### 1.4 Metabolismo energético em ovos

Os processos catabólicos, de uma forma geral, visam suprir os organismos de energia para manutenção de suas funções vitais. Esta energia, em seres heterotróficos, é retirada dos alimentos, que fornecem os carboidratos, os lipídeos e as proteínas. Já em seres autotróficos a energia da luz é utilizada para converter água e dióxido de carbono ( $\text{CO}_2$ ) em glicose. A utilização destas moléculas tem como objetivo principal a obtenção de energia na forma de ATP para as células. Quando o ATP é hidrolisado é capaz de liberar uma grande quantidade de energia, contida nas suas ligações de fosfato, permitindo que reações importantes, que *in vitro* são termodinamicamente desfavoráveis, aconteçam com muita facilidade dentro dos seres vivos. No entanto, dentro das células a degradação de moléculas

energéticas como a glicose não acontece em um único passo e sim, em múltiplas etapas reacionais coordenadas, denominadas vias metabólicas (Fothergill-Gilmore e Michels, 1993).

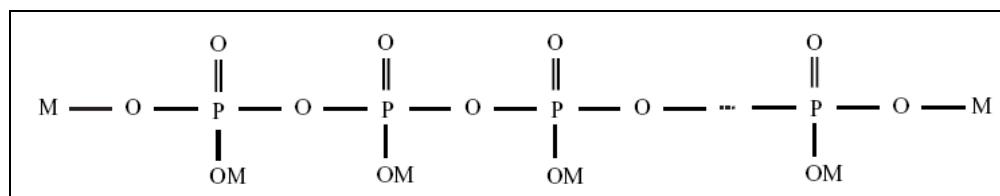
O custo metabólico no desenvolvimento embrionário pode ser definido como a quantidade total de energia consumida pelo embrião durante o desenvolvimento, incluindo o gasto para o crescimento, biossíntese e manutenção dos tecidos (Thompson e Stewart, 1997). A complexidade física e química do ovo é muito grande devido ao papel de proteção e manutenção do desenvolvimento embrionário neste sistema energeticamente fechado. Após a eclosão dos novos indivíduos, um dos fatores determinantes para a sua sobrevivência é a quantidade de vitelo remanescente da embriogênese (Sahoo et al., 1998).

Devido aos ovos não terem conexão com o organismo materno, consideramos que estes funcionam de forma análoga a um organismo em jejum, ou seja, já possuiriam todas as suas reservas necessárias para a manutenção e o suprimento energético. Os trabalhos relacionados ao estudo do desenvolvimento de embriões em ovíparos se direcionam, principalmente, para aspectos relacionados às estratégias de reservas aos novos indivíduos. O metabolismo protéico é o mais estudado, provavelmente, devido ao fato das proteínas serem os constituintes mais abundantes destes ovos (Campos et al., 2006, Anexo I).

Durante a ovogênese, a fêmea provê o ovo com ingredientes orgânicos e inorgânicos necessários para a construção do embrião (Stewart e Thompson, 1993). Alguns componentes extras, como o cálcio na casca e proteína na forma de albumina, podem ser adicionados depois da fertilização do ovo (Blackburn, 1998). Desta forma, no momento da ovoposição todos os componentes requeridos para o desenvolvimento do embrião já foram adicionados no ovo, com exceção de oxigênio para o metabolismo aeróbio (Rahn et al., 1974) e, em algumas espécies, água (Vleck, 1991). As reservas do ovo são compostas predominantemente de lipídeos e proteínas, mas também contém uma variedade de íons inorgânicos e vitaminas (Thompson e Stewart, 1997).

## 1.5 Polifosfatos

O fosfato inorgânico é essencial para todos os organismos, sendo requerido para vários processos metabólicos, tais como: biossíntese de ácidos nucléicos e fosfolipídeos, metabolismo energético e transdução de sinal. Sendo assim, os organismos necessitaram desenvolver um eficiente mecanismo regulatório para a sua aquisição, reserva e utilização. Há mais de cem anos atrás, o pesquisador Liberman, em 1890, descreveu a existência de polímeros de ortofosfatos em leveduras. Esses compostos são polímeros lineares contendo desde poucos a centenas de resíduos de ortofosfatos ligados por pontes do tipo fosfoanidrido. Eles apresentam uma fórmula geral  $M_{(n+2)}P_nO_{(3n+1)}$ , e seus ânions compreendem cadeias nas quais cada átomo de fósforo é ligado a seu vizinho através de átomos de oxigênio, formando assim uma estrutura linear que pode ser representada esquematicamente como mostrado na Figura 5 (Kornberg, 1995).



**Figura 5: Estrutura do polifosfato linear onde M é um  $H^+$  ou um cátion metálico.**

Inicialmente, os polifosfatos foram considerados como “fóssil molecular” ou somente como reserva de fósforo e de energia para a sobrevivência de organismos em condições severas. Evolutivamente, alguns autores sugerem que os polifosfatos possam ter tido uma origem abiótica (West e Ponnamperuma, 1970; Yamagata et al., 1991). Eles são gerados por desidratação simples de ortofosfatos em temperaturas elevadas, sendo encontrados em condensados vulcânicos (Brown e Kornberg, 2004). O estudo em procariontes primitivos tem mostrado um importante papel dos polifosfatos em seu metabolismo, onde foi observado que a glucoquinase tem uma atividade superior com polifosfatos do que com o ATP. Contradicoriamente, organismos mais evoluídos utilizam somente o ATP (Kulaev e Kulakovskaya, 2000). Modelos experimentais mostraram que os polifosfatos provavelmente

desempenharam um importante papel na síntese abiogênica de ácidos nucléicos e outras macromoléculas na Terra primordial (Kulaev e Vagabov, 1983). Na evolução pré-biótica, a abundância de polifosfatos como um polianion ou um agente fosforilador, poderia interagir com álcoois, açúcares, nucleosídeos, proteínas e com aminoácidos, gerando ácidos graxos e polipeptídeos. Dessa forma, os polifosfatos poderiam facilitar a orientação dos principais polímeros no mundo biótico: fosfolipídeos, ácidos nucléicos e proteínas (Brown e Kornberg, 2004).

Após evidências conclusivas de que estes compostos ocorrem em todos os organismos, incluindo os animais superiores (Tabela 1), se tornou óbvio que polifosfatos são necessários para praticamente todos os organismos vivos representativos de diferentes estágios de evolução (Kornberg, 1995).

**Tabela 1: Ocorrência de polifosfatos em várias células e tecidos<sup>a</sup>**

| Eucariotos                                         | Procariotos                                                                      |
|----------------------------------------------------|----------------------------------------------------------------------------------|
| Protozoários                                       | Bactéria                                                                         |
| Fungos<br><i>Saccharomyces cerevisiae</i> , 120 mM | <i>Escherichia coli</i> , 0,1 – 50 mM<br><i>Acinetobacter johnsonii</i> , 200 mM |
| Plantas                                            | Archaea                                                                          |
| Animais                                            | <i>Sulfolobus acidocaldarius</i> ,<br>0,5 – 1,5 mM                               |
| Fígado de rato, 26 µM                              |                                                                                  |
| Citosol, 12 µM                                     |                                                                                  |
| Núcleo, 89 µM                                      |                                                                                  |

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<sup>a</sup> Os níveis de polyP variam dependendo do estado metabólico da célula. Os valores dessa tabela foram obtidos sob condições metabólicas definidas e são úteis somente para ilustração. Retirado de Kornberg et al. (1999).

Em microorganismos onde se concentram a maioria dos estudos dessas moléculas tem sido mostrado que elas regulam vários processos bioquímicos tais como: metabolismo energético de P<sub>i</sub>, reserva e seqüestro de cátions, formação de canais de membrana, transporte de P<sub>i</sub>, envolvimento na formação e na função do envelope celular, regulação de genes e atividades de enzimas, envolvimento na virulência de alguns patógenos e ativação de proteases (Rashid et al., 2000; Kuroda et al., 2001; Kim et al., 2002; Nishii et al., 2005; Zhang et al., 2005; McInerney et al., 2006). Em eucariotos superiores algumas funções já foram descritas, tais como: ativação de cinases, envolvimento na

regulação da coagulação sanguínea, apoptose e proliferação de células cancerígenas de mamíferos (Lorenz et al., 1997; Wang et al., 2003; Hernandez-Ruiz et al., 2006; Kawano, 2006; Smith et al., 2006).

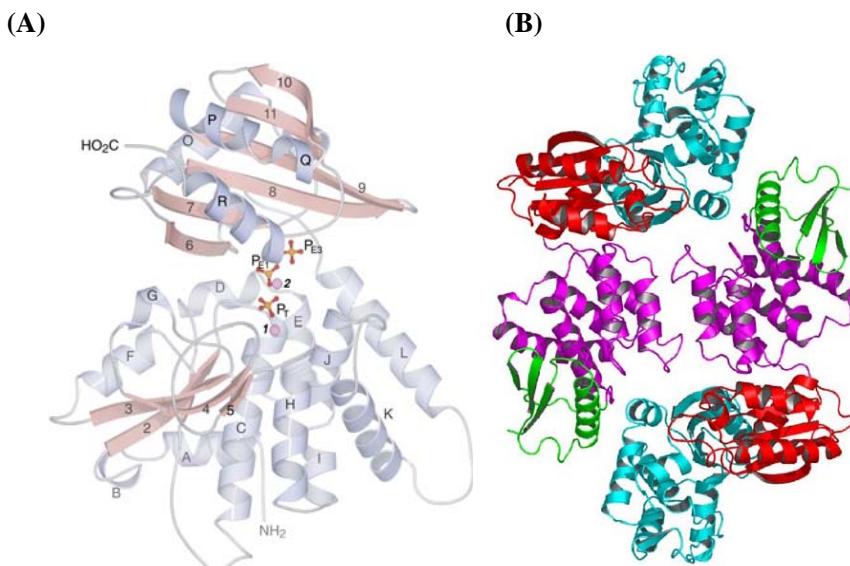
A hidrólise de polifosfatos é catalisada pelas exopolifosfatas e endopolifosfatas (Kulaev e Kulakovskaya, 2000). Exopolifosfatas são consideradas como enzimas regulatórias centrais do metabolismo de polifosfatos (Kulaev et al., 2000) e foram identificadas principalmente em diferentes organelas da célula eucariótica (Tabela 2). Em *Saccharomyces cerevisiae* foram caracterizados diferenças consideráveis do papel fisiológico para os polifosfatos em diferentes compartimentos celulares. No compartimento nuclear elas tem sido relacionadas com a regulação gênica, e no mitocondrial com a bioenergética celular (Kulaev e Kulakovskaya, 2000; Lichko et al., 2003).

**Tabela 2: Classificação das exopolifosfatas de *S. cerevisiae*. Retirado de Lichko et al. (2003).**

| Enzima         | Localização                     | Massa molecular (KDa) |
|----------------|---------------------------------|-----------------------|
| ExopolyPase 1  | Envelope celular, citosol       | 40                    |
| ExopolyPase 1a | Matriz mitocondrial             | 40                    |
| ExopolyPase 2  | Citoplasma, matriz mitocondrial | ~830                  |
| ExopolyPase 2a | Vacúolos                        | 245                   |
| ExopolyPase 3  | Membranas mitocondriais         | 120, 76               |
| ExopolyPase 4  | Núcleo                          | 57                    |

Baseado na estrutura primária, exopolifosfatas são classificadas em dois tipos: uma encontrada em fungos e protozoários pertencentes à superfamília das DHH fosfoesterases, que possuem um motivo Asp – His – His conservado (Aravind e Koonin, 1998), e uma outra presente em Eubactérias e Arqueobactérias pertencente à superfamília kinase/actina/hsp-70 (Reizer et al., 1993). Genes de ambos os tipos foram expressos em *Escherichia coli* (Akiyama et al., 1993; Wurst et al., 1995; Rodrigues et al., 2002; Kristensen et al., 2002) e a estrutura das enzimas de *E. coli* (Rangarajan et al., 2006) e *S. cerevisiae* (Ugochukwu et al., 2007) foram determinadas.

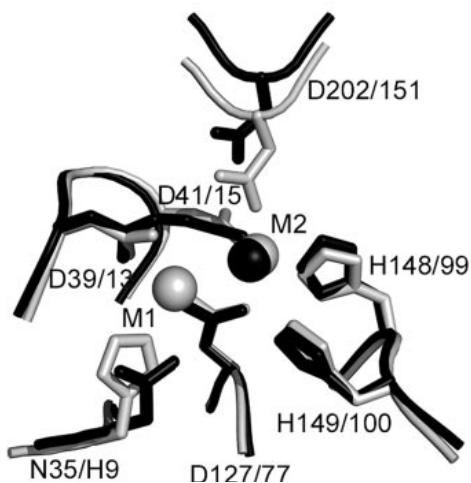
Em geral as duas famílias de exopolifosfatases não são similares. A de levedura possui dois domínios, enquanto a de *E. coli* possui quatro. Entretanto ambas possuem o sítio ativo localizado entre domínios conectados por um “link” flexível (Tammenkoski et al., 2007). Adicionalmente, as exopolifosfatases de *S. cerevisiae* e de *Aquifex aeolicus* são proteínas monoméricas (Figura 6A) (Lorenz et al., 1994; Wurst e Kornberg, 1994; Kristensen et al., 2004) e a de *E. coli* é dimérica (Figura 6B) (Akiyama et al., 1993; Rangarajan et al., 2006), sendo que *S. cerevisiae* possui até cinco diferentes exopolifosfatases em diferentes compartimentos (Lichko et al., 2003, vide tabela 2).



**Figura 6:** Estrutura geral das duas famílias de exopolifosfatases. (A) Estrutura do monômero da exopolifosfatase citoplasmática de *S. cerevisiae* com o seu sítio ativo localizado entre domínios conectados por um link flexível representado em  $P_T$ ,  $P_{E1}$  e  $P_{E3}$ . Retirado de Ugochukwu et al. (2007). (B) Estrutura do dímero da exopolifosfatase de *E. coli*. Retirado de Rangarajan et al. (2006).

A estrutura da exopolifosfatase de levedura possui similaridade com a família II das pirofosfatases, uma DHH fosfoesterase que catalisa uma reação similar com pirofosfato. Apesar de apresentar somente 12-17% de identidade, 11 dos 14 resíduos polares no sítio ativo da família II de pirofosfatase são conservadas nas exopolifosfatases (Figura 8) encontradas em fungos e protozoários, implicando em uma provável relação evolucionária (Merckel et al., 2001; Ahn et al., 2001). Embora existam similaridades funcionais e estruturais, a especificidade para substrato é bem diferente, a exopolifosfatase é muito

ativa na hidrólise de polifosfatos formados por três ou mais resíduos de fosfato, mas não hidrolisa pirofosfatos (Lorenz et al., 1994; Wurst e Kornberg, 1994). A pirofosfatase pertencente à família II hidrolisa pirofosfato, mas também possui uma pequena atividade contra polifosfatos (Parfenyev et al., 2001). Funcionalmente, exopolifosfatases de leveduras e de outros eucariontes têm sido preliminarmente caracterizadas, e os seus mecanismos de ação ainda não foram completamente elucidados (Tammenkoski et al., 2007).



**Figura 7: Comparação entre a exopolifosfatase da família DHH fosfoesterases e a família II das pirofosfatases: superposição do sítio ativo da exopolifosfatase de *S. cerevisiae* (em preto) e da pirofosfatase de *Streptococcus gordonii* (em cinza).**  
Figura retirada de Tammenkoski et al. (2007).

Polifosfatos são encontrados em todas as células desde o início da evolução, possuindo diversas funções específicas e cruciais para a sobrevivência celular, como por exemplo a capacidade de estimular a proliferação de células cancerígenas de mamíferos (Wang et al., 2003) e na participação com filamentos de actina nas operações celulares (Gomez-Garcia e Kornberg, 2004). Portanto, a sua vasta ocorrência e multifuncionalidade indicam que muitas importantes funções em distintos organismos ainda estão por serem descobertas possibilitando, neste caso, a ampliação do conhecimento da bioenergética celular e da embriogênese em artrópodes.

## OBJETIVOS

## 2      **Objetivos**

### **2.1    Objetivo Geral**

O presente trabalho tem por objetivo analisar o metabolismo de polifosfatos inorgânicos durante o desenvolvimento embrionário do carrapato *R. microplus*, com o intuito de avaliar o papel destas moléculas neste processo de desenvolvimento.

### **2.2    Objetivos Específicos**

- Avaliar o papel dos polifosfatos totais e sua possível relevância como reserva de fosfato para o embrião e/ou participação em processos regulatórios durante o desenvolvimento embrionário do *R. microplus*.
- Analisar o metabolismo dos polifosfatos na fração nuclear e mitocondrial, avaliando o seu papel durante o desenvolvimento embrionário do *R. microplus*.

## **MATERIAIS E MÉTODOS**

### 3 Materiais e Métodos

#### 3.1 Animais

Foram utilizados carapatos da espécie *Rhipicephalus (Boophilus) microplus* criados em bovinos na Faculdade de Veterinária do Rio Grande do Sul. Os hospedeiros foram mantidos isolados em estábulos, ou seja, sem contato com o campo. Após 21 dias da colocação das larvas no dorso do bovino, período no qual ocorre a queda das teleóginas, o estábulo foi lavado e as fêmeas adultas coletadas com auxílio de peneiras, sendo utilizadas para postura dos ovos que foram usados nos experimentos.

Os ovos foram coletados a partir de fêmeas adultas ingurgitadas, sendo que os dias de desenvolvimento do embrião são contados a partir da ovoposição, e foram mantidos à temperatura de 28º C e umidade de 80%.

#### 3.2 Extração e quantificação de polifosfatos

A extração de polifosfatos foi realizada de acordo com a metodologia de Clark et al. (1986) a partir de homogenato de ovos, fração de núcleo e mitocôndria de diferentes dias após a ovoposição, em triplicata. Foram adicionados 200 µL de tampão de extração (Tris-HCl 50 mM pH 7,5 Uréia 1 M, SDS 0,5% e EDTA 10 mM) acrescidos de 300 µL de TCA 2%, e o material foi centrifugado a 11000 X g por 10 min. O precipitado foi ressuspenso em 1 mL de TCA/acetona 0,7-67% (v/v) e centrifugado a 11000 X g por 10 min. Em seguida, o precipitado obtido foi lavado com acetona 67%, ressuspenso em 0,8 mL de EDTA 2 mM, e teve o pH ajustado entre 7-8. Então foram adicionados 0,3 mL de metanol/clorofórmio (1/1,v/v, saturado com  $(\text{NH}_4)_2\text{SO}_4$  0,1 M, pH 6,5 e a amostra mantida “overnight” a -20º C. No dia seguinte, a amostra foi centrifugada a 11000 X g por 5 min e o sobrenadante foi transferido para outro tubo. O precipitado foi tratado com 0,8 mL de EDTA 2 mM e 0,3 mL de clorofórmio. Quantidades residuais de DNA e RNA foram removidos por tratamento com DNase e RNase (250 µg/mL cada), na presença de  $\text{MgCl}_2$  1mM, por 1 h, a 37º C.

Para a quantificação foram adicionados 10 µL da amostra de polifosfato em 0,35 mL de ácido acético 0,2 N e 0,35 mL de azul de toluidina 30 mg/L. A

quantidade de polifosfato foi determinada dentro de 15 min por comparação com a curva padrão produzida usando 1 a 5 µg de poly P<sub>65</sub> (Sigma type 65) a 630nm (Lorenz *et al.*, 1997).

### **3.3 Isolamento da fração nuclear e mitocondrial**

O isolamento da fração nuclear e mitocondrial foi realizada de acordo com a metodologia de Campos et al. (2007) descrito no anexo III, onde um grama de ovos foram homogeneizados em um tampão de isolamento contendo sacarose 0,5 M, leupeptina 1 µM, pepstatina 10 µM, MgCl<sub>2</sub> 20 mM, EGTA 10 mM, albumina bovina livre de ácidos graxos 1%, e HEPES 50 mM (pH 7,2). O homogenato foi centrifugado a 8000 X g por 15 min, a 4° C. O sobrenadante foi então removido e o precipitado foi ressuspenso no tampão de homogeneização e centrifugado a 500 X g por 5 min, a 4° C. O sobrenadante foi novamente removido e centrifugado a 2000 X g por 10 min, a 4° C para a precipitação da fração nuclear. O sobrenadante foi submetido a uma nova centrifugação a 7000 X g por 15 min, a 4° C, onde o precipitado corresponde à fração mitocondrial, que foi ressuspensa em tampão contendo KCl 120 mM, EGTA 1 mM, albumina bovina livre de ácidos graxos 0,2% e HEPES 3 mM (pH 7,2).

### **3.4 Isolamento da facção de membrana e solúvel mitocondrial**

Para o isolamento da facção de membrana e solúvel mitocondrial foram adicionados 500 µL de água milliQ para o rompimento das mitocôndrias no pellet mitocondrial e em seguida adicionado 500 µL de tampão de isolamento duas vezes concentrado. Foi realizada então uma centrifugação a 100000 X g por 40 min, a 4° C, onde o precipitado corresponde à fração de membranas e o sobrenadante à fração solúvel.

### **3.5 Extração e quantificação de RNA total**

A extração e quantificação de RNA total foi realizada de acordo com a metodologia de Sambrook et al. (1989). Um grama de ovos foi macerado em nitrogênio líquido, e em seguida a amostra foi homogeneizada em 10 mL de Trizol. A amostra foi centrifugada a 12000 X g por 10 min, a 4° C, o material insolúvel foi descartado, e ao sobrenadante foram adicionados 500 µL de

clorofórmio. Em seguida, o material foi homogeneizado por inversão, incubado a temperatura ambiente por 3 min, e submetido a centrifugação de 12000 X g por 15 min, a 4° C. O sobrenadante foi recolhido e adicionado 500 µL de isopropanol. Posteriormente, a amostra foi homogeneizada por inversão e incubada a -20 ° C por 2 hs. Em seguida, a amostra foi centrifugada a 12000 X g por 15 min a 4° C, o sobrenadante descartado e o precipitado lavado com etanol 75%, e após a secagem foi ressuspendido em água DEPC. Foi medida a absorbância a 260 e 280 nm para determinar a pureza e para a quantificação foi aceito que 1 unidade de absorbância a 260 nm corresponde a 40 µg/mL de RNA.

### **3.6 Quantificação de ortofosfato**

Dez miligramas de ovos de diferentes dias após a ovoposição, em triplicata, foram homogeneizados em 500 µL de água destilada e centrifugados a 200 X g por 1 min. Foram tomadas alíquotas de 100 µL e adicionadas em 1 mL de solução de coloração feita na hora do ensaio pela mistura da solução I que é composta de, SDS 0,5%, NH<sub>4</sub>MoO<sub>4</sub> 0,5% e H<sub>2</sub>SO<sub>4</sub> 2% com a solução II que é feita de ácido ascórbico 0,5%. Em seguida foram quantificadas em espectrofotômetro (Shimadzu UV-visível - 1240) a 750 nm. O conteúdo de ortofosfato nos ovos foi calculado com base em uma curva padrão de fosfato de sódio submetida às mesmas condições de ensaio (Fiske e Subbarow, 1925).

### **3.7 Análise eletroforética de polifosfato**

A análise eletroforética de polifosfato após a extração foi realizada de acordo com Clark et al. (1986). Gel de poliacrilamida – Uréia foi preparado misturando 10,51 g de uréia, 3,75 mL de solução de acrilamida e 2,5 mL de Tris-borato 0,9 M, (pH 8,3) e EDTA 27 mM para um volume final de 25 mL. Foi feita uma pré-corrida de 300 V por 1h, e em seguida o poly P foi misturado com 0,25 volume de tampão de amostra composto de sacarose 50%, azul de bromofenol 0,125% e Tris-borato 450 mM (pH 8,3), EDTA 13,5 mM, e aplicado no gel. A corrida foi realizada a 300 V até que o marcador (azul de bromofenol) tivesse migrado 10 cm, e o gel foi corado com azul de toluidina 0,05%, metanol

25% e glicerol 5% por 20 min, e em seguida descorado com o mesmo solvente, sem o azul de toluidina.

### **3.8 Ensaio da exopolifosfatase**

As amostras foram adicionadas em 100 µL tampão Tris-HCl 50 mM (pH 7,4) contendo MgCl<sub>2</sub> 5 mM e foram incubadas a 30° C por 15 min. Foram utilizados poly P<sub>3</sub>, poly P<sub>15</sub> e poly P<sub>65</sub> 3 mM como substratos e heparina como inibidor. O P<sub>i</sub> formado durante a reação foi determinado espectrofotometricamente adicionando molibdato de amônio 0,5%, ácido sulfúrico 0,35 M, SDS 0,5 M e ácido ascórbico 10%. A absorbância foi medida a 750 nm após 15 min de incubação. Foi definido como 1 unidade de atividade enzimática a quantidade de enzima capaz de liberar 1 µmol de P<sub>i</sub> por min. A concentração de proteína foi determinada de acordo com Bradford, (1976), utilizando albumina bovina como padrão.

### **3.9 Atividade da exopolifosfatase durante a respiração mitocondrial**

A atividade da exopolifosfatase foi determinada nas mitocôndrias isoladas dos ovos utilizando piruvato e succinato como substratos oxidativos, a 28° C, em 150 µL de meio de incubação com 0,5 mg de proteínas de mitocôndrias. O meio de reação continha poly P<sub>15</sub> 5 mM, KCl 120 mM, EGTA 1 mM, MgCl<sub>2</sub> 5 mM, piruvato ou succinato 3 mM e ADP 0,2 mM em Tris-HCl 50 mM (pH 7,4), e foi usado KCN 1 mM como inibidor da cadeia respiratória. A atividade da exopolifosfatase foi determinada após 15 min de incubação (Pestov *et al.*, 2004).

### **3.10 Parâmetros respiratórios**

A taxa de consumo de O<sub>2</sub> foi determinada usando um eletrodo de Clark (YSI, mod. 5775, YellowSprings, OH). As medidas foram realizadas em 1,75 mL de meio de reação, a 28° C, contendo KCl 120 mM, EGTA 1 mM, albumina bovina livre de ácidos graxos 0,2%, HEPES 3 mM (pH 7,2), KH<sub>2</sub>PO<sub>4</sub> 2,5 mM, e

KCN 1 mM ou oligomicina 2,5 µg/mL como inibidor. Foi adicionado 0,5 mg de proteína de mitocôndrias, e após 1 min de estabilização a reação foi iniciada com a adição de piruvato 5 mM. A razão do controle respiratório (RCR) foi definida como a taxa de consumo de oxigênio estimulado por ADP (estado 3) dividido pela taxa de respiração determinada na presença de oligomicina (estado 4<sub>o</sub>), um inibidor da ATP sintase. Apesar do estado 4<sub>o</sub> não ser equivalente ao estado 4 clássico, que é a taxa obtida após pequenas quantidades de ADP serem quase completamente convertidas a ATP, o uso de oligomicina elimina a re-síntese de ATP que foi degradado por contaminação com ATPases durante o estado 4 (Kristian et al., 2006).

### **3.11 Consumo de O<sub>2</sub> utilizando poly P como doador de P<sub>i</sub>**

O consumo de oxigênio utilizando-se poly P como fonte de P<sub>i</sub> foi determinado a 28° C em 1,75 mL de meio de reação com 0,5 mg de mitocôndria considerando a quantidade de proteínas em mg/mL. O meio de reação consistia de KCl 120 mM, EGTA 1 mM, albumina livre de ácidos graxos 0,2 % em HEPES 3 mM (pH 7,2). Após 1 min de estabilização, a respiração mitocondrial foi iniciada adicionando 5 mM de piruvato e 0,2 mM de ADP. Durante a respiração mitocondrial foram adicionados 5 mM de poly P<sub>15</sub> e 5 mM de KH<sub>2</sub>PO<sub>4</sub>. A concentração de oxigênio no meio foi determinada com auxílio de um eletrodo de Clark pela inclinação da curva obtida em oxígrafo (Yellow Springs Instruments Co.).

### **3.12 Atividade da F-ATPase**

A atividade F-ATPase sensível a azida foi determinada espectrofotometricamente de acordo com Li e Neufeld (2001). O ensaio foi realizado a 30° C em uma reação contendo Tris-HCl 50 mM pH 8,0, MgCl<sub>2</sub> 2 mM, KCl 100 mM e ATP 2 mM. Foi usada azida 5 mM como inibidor. Foi definido como 1 unidade de atividade enzimática a quantidade de enzima capaz de liberar 1 µmol de P<sub>i</sub> por min.

### **3.13 Atividade da glicose-6-fosfato desidrogenase**

A amostra foi adicionada em meio de reação contendo Tris-HCl 50 mM pH 7,4,  $\beta$ NAD<sup>+</sup> 6 mM e glicose 6 fosfato 100 mM. A atividade a 30° C foi determinada espectrofotometricamente a 340 nm pela taxa de formação de NADPH de acordo com Worthington (1988).

### **3.14 Estudos cinéticos**

Os parâmetros cinéticos, como  $K_m$  e  $V_{max}$  da exopolifosfatase, foram determinados por regressão não linear usando o programa GraphPad Prism. As determinações foram realizadas com concentrações de substratos numa escala de 0,2 a 2  $K_m$ s. Foram usados como substratos poly P<sub>3</sub>, poly P<sub>15</sub> e poly P<sub>65</sub>, e como inibidor foi usada heparina.

## **RESULTADOS**

#### 4 Resultados

A embriogênese de *R. microplus* é um processo complexo que envolve o acúmulo de diversas reservas estratégicas. A cinética de utilização de macromoléculas como lipídeos, proteínas, carboidratos e RNA durante a embriogênese do *R. microplus* apresentam importantes correlações com eventos morfológicos da embriogênese deste carapato, possibilitando correlacionar e entender outros fenômenos que estão ocorrendo em fases marcantes do desenvolvimento.

Previamente aos estudos do metabolismo de polifosfatos, foi realizada uma análise dessas reservas estratégicas durante a embriogênese do *R. microplus* (Campos et al., 2006, Anexo I). Verificou-se que os carboidratos e os lipídeos totais são mobilizados durante a primeira fase da embriogênese. Temos considerado como primeira fase da embriogênese os estágios do 1º até o 9º dia do desenvolvimento, que passa pelo processo de formação de um blastoderma celular no 5º dia e posterior segmentação, entre o 7º e o 9º dia. Observa-se então que o consumo de lipídeos ocorreu durante a formação do blastoderma celular (Campos et al., 2006, Figura 4A do Anexo I) e o de carboidratos durante a segmentação do embrião (Campos et al., 2006, Figura 4B do Anexo I).

A vitelina do *R. microplus* foi previamente isolada e caracterizada (Logullo et al., 2002). Neste trabalho foi visto que há uma queda de aproximadamente 15% do conteúdo de vitelina nos quatro primeiros dias, e de 20% do 8º dia até a eclosão dos ovos no 19º dia. Se considerarmos que a vitelina pode representar até 90% do conteúdo protéico dos ovos destes animais, e que ela é a proteína de reserva mobilizada neste processo para a formação do embrião, pode-se dizer que o seu consumo representa a mobilização deste substrato no ovo. Para averiguar este fenômeno, foi visto como ocorre a mobilização das proteínas totais do embrião. Interessantemente, o conteúdo de proteínas totais permaneceu constante durante toda a embriogênese apesar de ocorrer um consumo em torno de 40% da vitelina, possibilitando à larva eclodir com 60% do conteúdo de vitelo inicial (Campos et al., 2006, Figura 5 do Anexo I).

A variação do RNA total durante a embriogênese apresenta uma relação direta com a taxa metabólica dos embriões. O conteúdo de RNA total permaneceu quase constante nos primeiros três dias, tendo um rápido aumento a partir da formação do blastoderma celular para então permanecer nestes patamares até próximo à eclosão (Campos et al. (2006), Figura 4C do Anexo I).

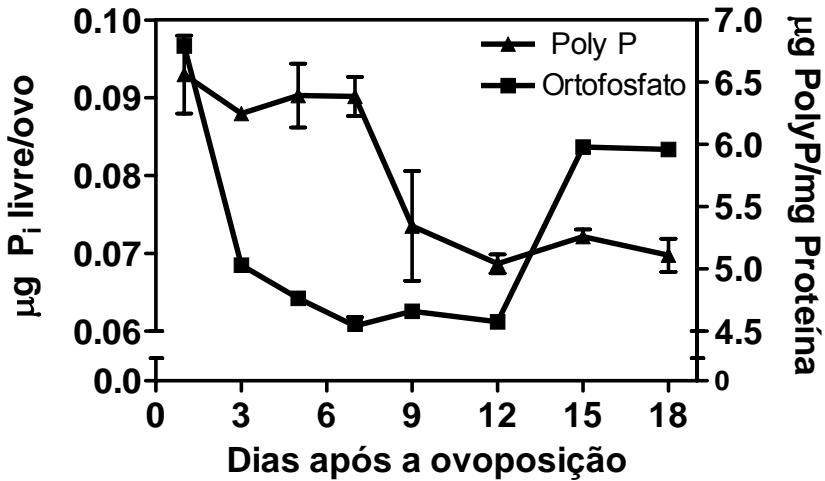
#### **4.1 Caracterização do conteúdo de polifosfato total na embriogênese do *R. microplus*<sup>\*</sup>**

Para o estudo do metabolismo de moléculas de polifosfatos durante a embriogênese do *R. microplus*, inicialmente foi analisado o conteúdo de polifosfato total e de ortofosfato, o tamanho das cadeias de polifosfato e a atividade exopolifosfatásica no homogenato total.

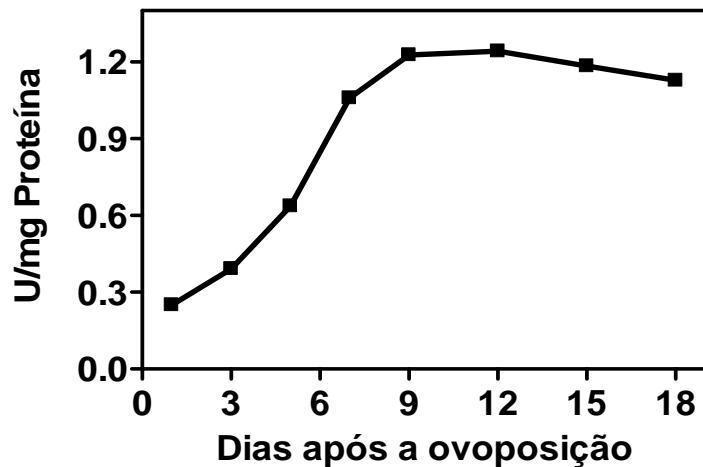
Os maiores níveis de polifosfato total foram detectados durante a celularização e segmentação do embrião, do 5º ao 7º dia de desenvolvimento. Após esse período ocorre uma diminuição até o 9º dia e mantém-se estável até o fim da embriogênese. O conteúdo de ortofosfato diminui rapidamente durante a formação do blastoderma sincicial, no 3º dia de desenvolvimento, mantendo-se estável até o 12º dia, onde ocorre um rápido aumento até próximo à eclosão (Figura 8). No homogenato total a atividade da exopolifosfatase é crescente até o 12º dia, permanecendo estável até próximo da eclosão (Figura 9). O tamanho das cadeias de polifosfato foi comparada durante o desenvolvimento embrionário constatando o aparecimento de cadeias de polifosfato cada vez menores a partir da segmentação do embrião, se comparada com o início do desenvolvimento (Figura 10).

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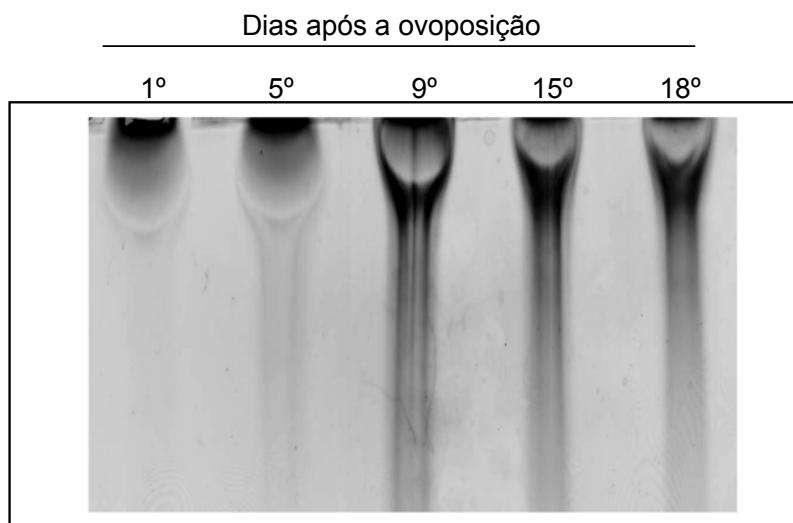
\* Os resultados deste tópico foram publicados no artigo em Anexo II Campos et al., (2008).



**Figura 8:** Conteúdo de polifosfato total (Poly P) e de ortofosfato na embriogênese de *R. microplus*. Os dados expressam a média  $\pm$  desvio padrão de 3 ensaios, sendo cada ensaio realizado em triplicata.



**Figura 9:** Atividade da exopolifosfatase em homogenato total ao longo da embriogênese de *R. microplus*. Os dados expressam a média  $\pm$  desvio padrão de 3 ensaios, sendo cada ensaio realizado em triplicata.



**Figura 10:** Análise eletroforética em gel de poliacrilamida das cadeias dos polifosfatos no 1º, 5º, 9º, 15º e 18º dia após a ovoposição de *R. microplus*.

#### 4.2 Metabolismo nuclear e mitocondrial de polifosfatos\*

Para a análise do metabolismo nuclear e mitocondrial dos polifosfatos foi realizado um fracionamento celular no qual necessitou-se de grandes quantidades de ovos frescos (2 g) para a obtenção da fração nuclear e mitocondrial ativa. Foram usados ovos no estágio de segmentação, no 9º dia após a ovoposição, e a pureza e a integridade da fração nuclear foram avaliadas através de análise por microscopia de contraste de fase (resultado não mostrado). A fração nuclear também foi caracterizada bioquimicamente pela ausência de marcadores de outros compartimentos, não sendo detectada nenhuma atividade da F-ATPase, sensível a azida, um marcador mitocondrial, e da glicose-6-fosfato desidrogenase, um marcador citoplasmático. Na fração mitocondrial, quando oxidando piruvato, o consumo de O<sub>2</sub> foi de 29 nmol O<sub>2</sub>/min.mg de proteína e o RCR foi 6,8. Este processo foi sensível a KCN e oligomicina e apresentou hidrólise de ATP pela F-ATPase maior que 80% (Tabela 3).

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\* Os resultados deste tópico foram publicados no artigo em Anexo II Campos et al., (2008).

Tabela 3: Caracterização do fracionamento celular de embriões de *R. micropus* no 9º dia de desenvolvimento.

|                     | ATPase<br>(U / mg proteína) | G6PDH*<br>(U / mg proteína) | O <sub>2</sub> consumption<br>(nmol min <sup>-1</sup> mg proteína <sup>-1</sup> ) | RCR* | F - ATPase |
|---------------------|-----------------------------|-----------------------------|-----------------------------------------------------------------------------------|------|------------|
| Homogenato          | 0.7                         | 2.9                         | —                                                                                 | —    | 49.5 %     |
| Fração nuclear      | —                           | —                           | —                                                                                 | —    | —          |
| Fração mitocondrial | 1.5                         | —                           | 29.7                                                                              | 6.8  | 83.4 %     |

\*G6PDH = glicose-6-fosfato desidrogenase

\*RCR = razão do controle respiratório

Também foram determinados, durante a embriogênese, o conteúdo de polifosfatos e a atividade da exopolifosfatase nas frações de núcleo e mitocôndria. Observou-se que a atividade da exopolifosfatase nuclear aumentou ao longo do desenvolvimento embrionário, com decréscimo no conteúdo de polifosfatos, refletindo a atividade da enzima (Figura 11A). A fração mitocondrial apresentou uma mobilização de polifosfato diferente, tendo maior atividade da exopolifosfatase e menores níveis de polifosfato durante a formação do blastoderma celular e segmentação do embrião, entre o 5º e 7º dia de desenvolvimento (Figura 11B).

A influência do RNA total na atividade da exopolifosfatase nuclear e mitocondrial foi investigada entre concentrações de 0,3 a 1,4 µg de RNA endógeno. Verificou-se que a exopolifosfatase nuclear apresentou um estímulo em torno de duas vezes, enquanto a enzima mitocondrial foi insensível ao RNA (Figura 12).

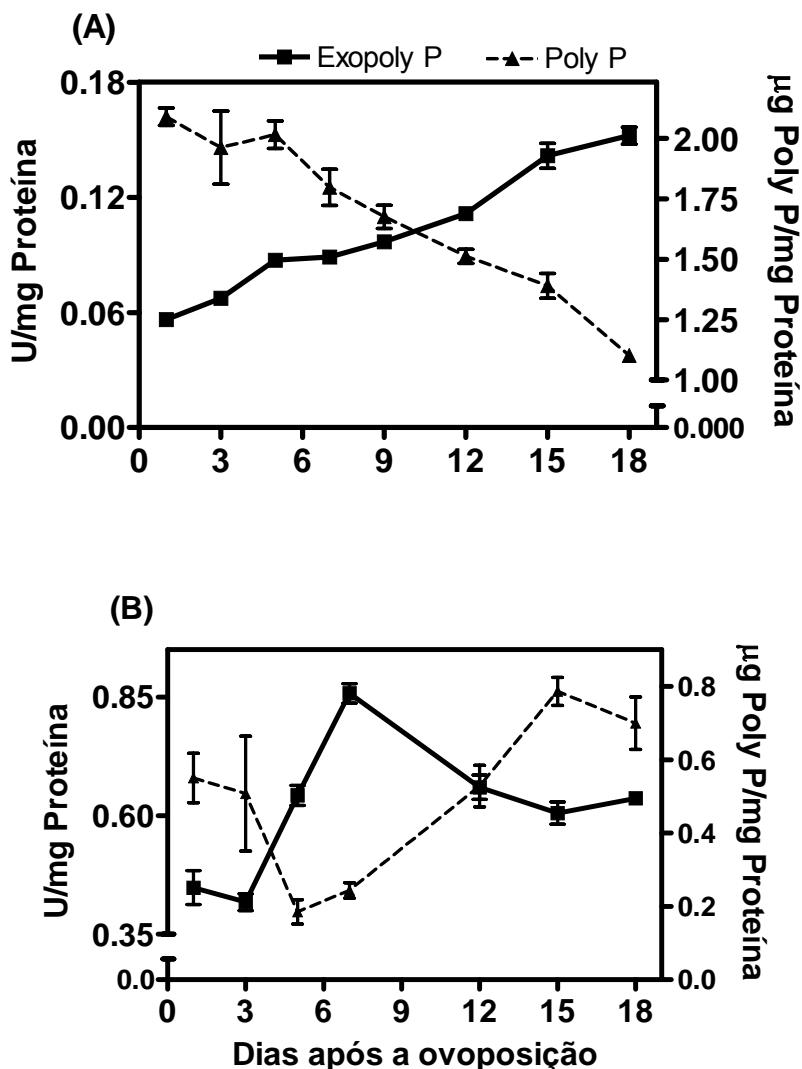
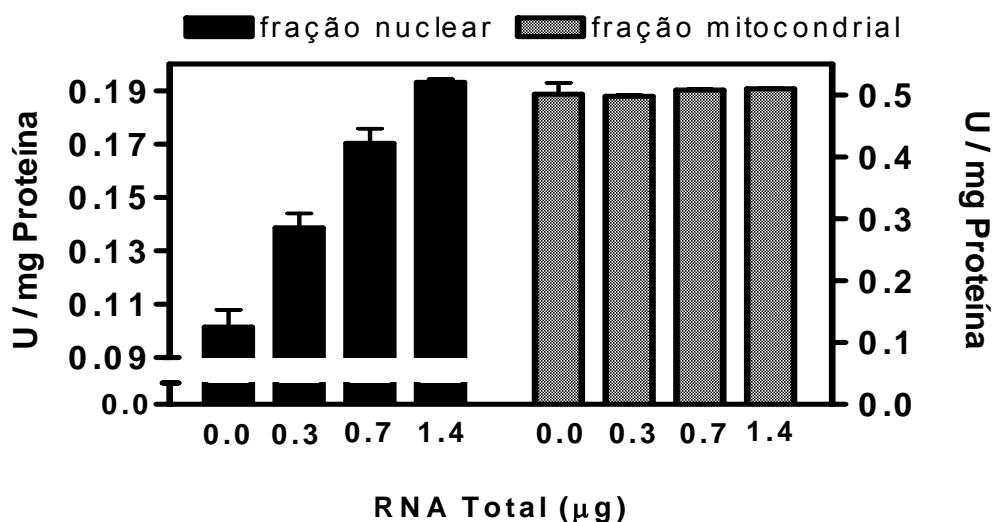


Figura 11: Atividades da exopolifosfatase (Exopoly P) e conteúdo de polifosfato (Poly P) nuclear (A) e mitocondrial (B) durante a embriogênese de *R. microplus*. Os dados expressam a média  $\pm$  desvio padrão de 3 ensaios, sendo cada ensaio realizado em triplicata.



**Figura 12:** Influência do RNA total endógeno na atividade da exopolifosfatase nuclear e mitocondrial em ovos no estágio de segmentação, 9º dia de desenvolvimento. Os dados expressam a média ± desvio padrão de 3 ensaios, sendo cada ensaio realizado em triplicata.

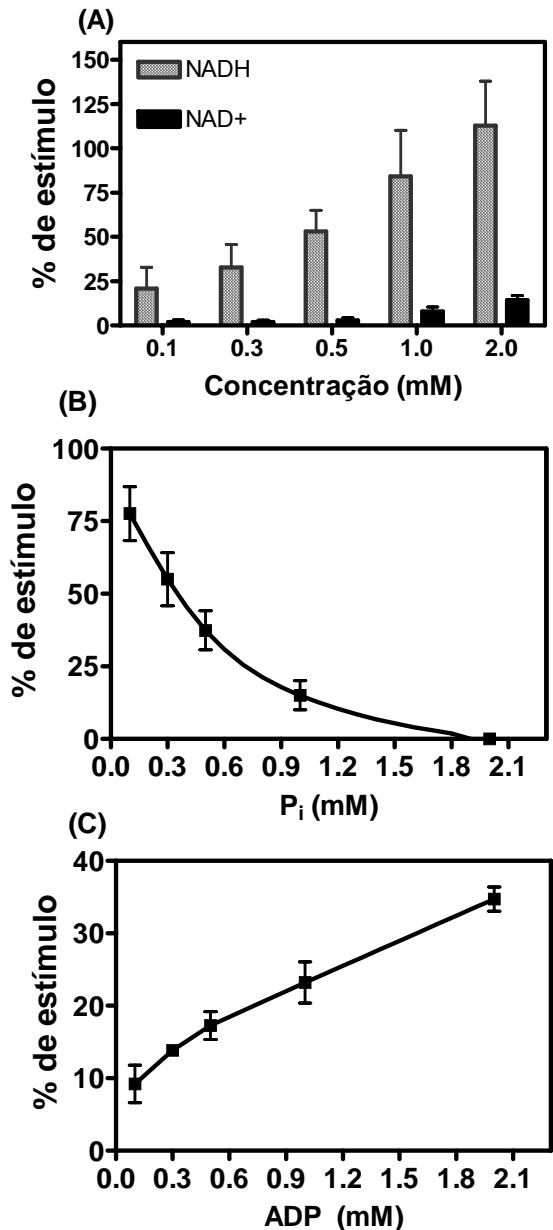
#### 4.3 Modulação da atividade exopolifosfatásica mitocondrial por demanda de $P_i^*$

Mitocôndrias de ovos no estágio de segmentação, no 9º dia de desenvolvimento, foram isoladas, e a relação da exopolifosfatase mitocondrial com o metabolismo energético da célula foi determinada. Para isto, foi investigada a influência de NADH,  $NAD^+$ ,  $P_i$  e ADP em concentrações variando entre 0,1 e 2,0 mM. Observou-se que a enzima foi estimulada em torno de duas vezes por NADH, enquanto  $NAD^+$  pouco influenciou na atividade (Figura 13A). O  $P_i$  inibiu completamente (Figura 13B), ao passo que ADP também estimulou a atividade da enzima, porém não tanto quanto observado para o NADH (Figura 13C).

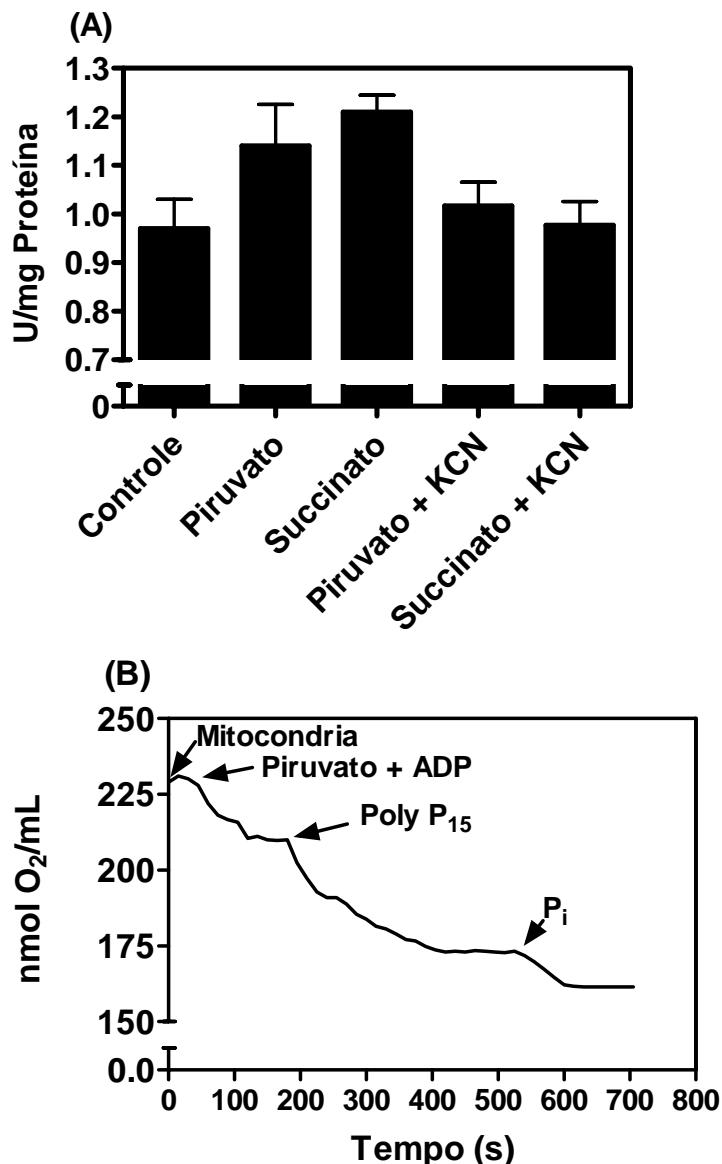
A atividade da exopolifosfatase mitocondrial foi medida durante a respiração celular, usando piruvato ou succinato como substratos, e o consumo de  $O_2$  foi monitorado, usando polifosfato como única fonte de  $P_i$ . Verificou-se que a atividade da enzima aumentou em torno de 17 e 25% usando piruvato e succinato, respectivamente. Quando a respiração mitocondrial foi inibida por KCN, o efeito estimulatório de ambos os substratos desapareceu (Figura 14A). A adição de poly  $P_{15}$  na concentração de 5 mM estimulou o consumo de  $O_2$  e quando um novo estado 4 foi estabelecido, o  $P_i$  na concentração de 5 mM foi adicionado estimulando o consumo de  $O_2$  novamente (Figura 14B).

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\* Os resultados deste tópico foram publicados no artigo em Anexo III Campos et al., (2007).



**Figura 13:** Efeito do NADH e NAD<sup>+</sup> (A), P<sub>i</sub> (B) e ADP (C) na atividade da exopolifosfatase mitocondrial em ovos de *R. microplus* no estágio de segmentação, no 9º dia de desenvolvimento. Os dados expressam a média ± desvio padrão de 3 ensaios, sendo cada ensaio realizado em triplicata.



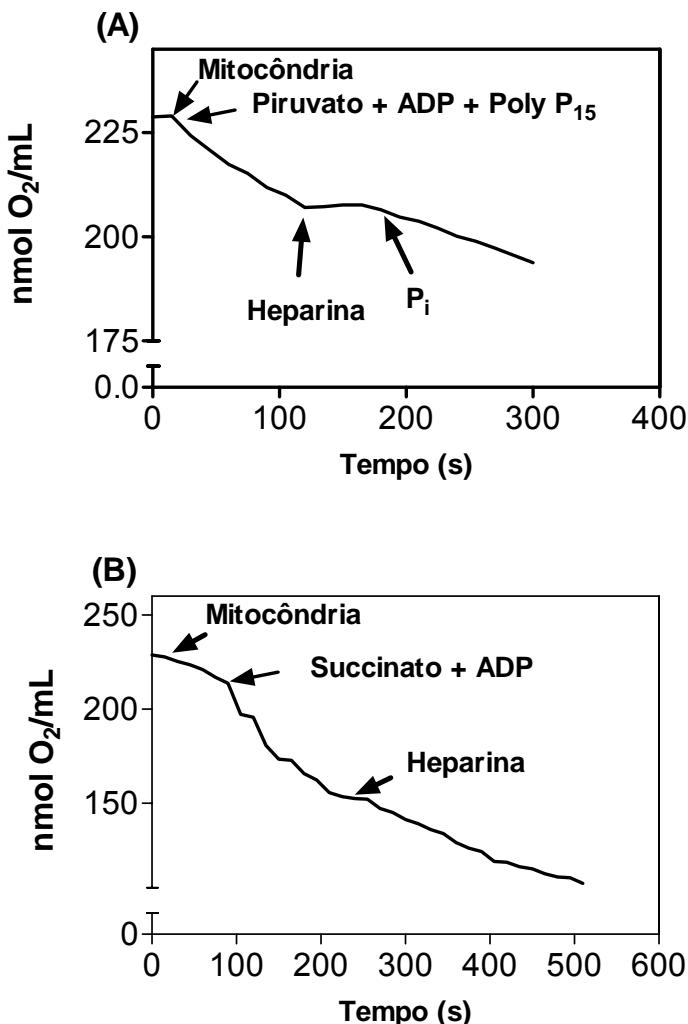
**Figura 14:** Efeito do piruvato, succinato e KCN na atividade da exopolifosfatase durante a respiração mitocondrial (A) e consumo de O<sub>2</sub> utilizando polifosfato como único doador de P<sub>i</sub> (B) em ovos de *R. microplus* no estágio de segmentação, no 9º dia de desenvolvimento. Os dados em (A) expressam a média ± desvio padrão de 3 ensaios, sendo cada ensaio realizado em triplicata.

#### **4.4 Isolamento da fração de membrana e solúvel mitocondrial**

Para entender melhor o papel da exopolifosfatase mitocondrial foi isolada a fração de membrana e a fração solúvel mitocondrial e determinada a sua localização. A maior atividade específica foi detectada na fração de membrana, apresentando também uma atividade na fração solúvel, porém menor (Tabela 5). A heparina, único inibidor descrito para exopolifosfatases, foi capaz de bloquear a ativação da respiração celular por polifosfato, mostrando a importância da exopolifosfatase de membrana para a respiração mitocondrial (Figura 15A). Como controle, foi visto que a heparina afeta um pouco a respiração mitocondrial, porém não é capaz de inibi-la completamente (Figura 15B).

Tabela 5: Isolamento da Fração Solúvel e de Membrana Mitocondrial

|                    | Atividade<br>(U) | Proteína<br>(μg) | Atividade<br>(U / mg proteína) |
|--------------------|------------------|------------------|--------------------------------|
| Mitocôndria        | 16,4             | 22,4             | 0,7                            |
| Fração de Membrana | 4,7              | 13,3             | 0,3                            |
| Fração Solúvel     | 10,2             | 7,18             | 1,42                           |



**Figura 15:** Efeito da Heparina no consumo de O<sub>2</sub> utilizando poly P como doador de P<sub>i</sub> (A) e na adição de P<sub>i</sub> exógeno (B) em ovos de *R. microplus* no estágio de segmentação, no 9º dia de desenvolvimento.

#### 4.5 Caracterização cinética da exopolifosfatase nuclear e mitocondrial\*

As exopolifosfases nuclear e mitocondrial foram comparadas cineticamente em função de suas atividades e afinidades. O  $K_{map}$  foi medido usando polifosfato de 3 fosfatos (poly P<sub>3</sub>), polifosfato de 15 fosfatos (poly P<sub>15</sub>) e polifosfato de 65 fosfatos (poly P<sub>65</sub>) como substratos em ovos no estágio de segmentação, 9º dia de desenvolvimento. A enzima mitocondrial apresentou uma afinidade muito maior pelo poly P<sub>3</sub> do que pelo outros substratos, em torno de 10 vezes. Por outro lado, a enzima nuclear apresentou praticamente a mesma afinidade por todos os substratos analisados (Tabela 4). Também foi analisada a influência de cátions divalentes, onde a hidrólise de polifosfato estava de acordo com a cinética de Michaelis, sendo que a exopolifosfatase nuclear foi estimulada por Mg<sup>+2</sup> 2,5 mM e Co<sup>+2</sup> 0,1 mM, embora a mitocondrial tenha sido estimulada somente por Mg<sup>+2</sup> (Figura 16A). A heparina, o principal inibidor descrito para exopolifosfatas, foi efetivo em ambas as frações, inibindo-as quase que completamente (Figura 16B).

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\*Os resultados deste tópico foram publicados no artigo em Anexo II Campos et al., (2008)

Tabela 4: Valores de  $K_m$  aparente da exopolifosfatase na fração nuclear e mitocondrial em ovos de *R. micropus* no 9º dia de desenvolvimento

| Substrato            | Fração Nuclear |                         | Fração Mitocondrial |
|----------------------|----------------|-------------------------|---------------------|
|                      |                | $K_m$ ( $\mu\text{M}$ ) |                     |
| Poly P <sub>3</sub>  | 0,7            | 0,2                     |                     |
| Poly P <sub>15</sub> | 1,1            | 2,8                     |                     |
| Poly P <sub>65</sub> | 0,9            | 3,6                     |                     |

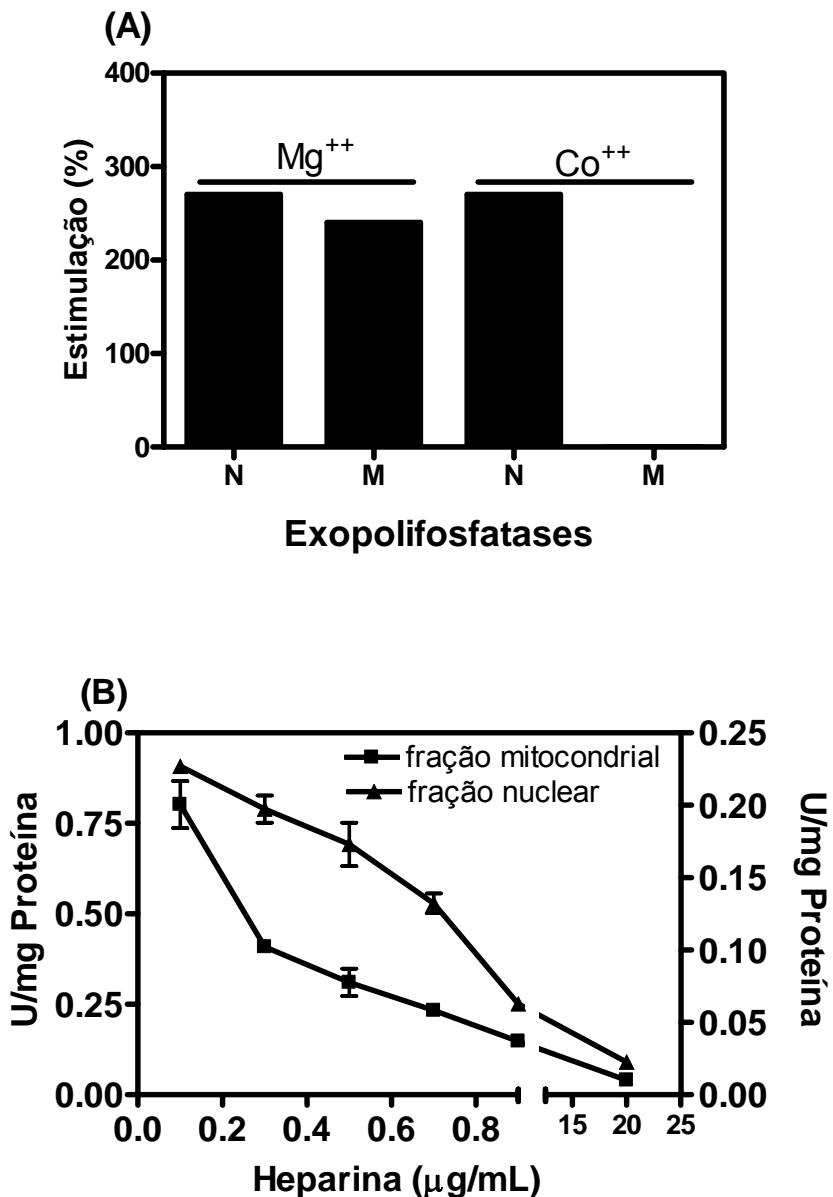


Figura 16: Efeito de cátions divalentes Mg<sup>++</sup> e Co<sup>++</sup> (A) e heparina (B) na atividade da exopolipofatase nuclear (N) e mitocondrial (M) em ovos de *R. microplus* no estágio de segmentação, no 9º dia de desenvolvimento. Os dados expressam a média ± desvio padrão de 3 ensaios, sendo cada ensaio realizado em triplicata.

## DISCUSSÃO

## 5 Discussão

Embora a primeira evidência da presença de polifosfatos em células de mamíferos ter sido obtida na década de 70 (Gabel e Thomas, 1971), o metabolismo deste biopolímero em eucariontes superiores ainda é pouco estudado. Com o crescente interesse em caracterizar as funções dos polifosfatos em eucariontes superiores surgem importantes relatos sobre o assunto, conforme foi descrito para diferentes frações subcelulares e órgãos em roedores (Kumble e Kornberg, 1996). Também existem relatos recentes sobre a presença de polifosfatos em sangue humano e no tecido ósseo (Leyhausen et al., 1998; Smith et al., 2006). Entretanto não foi encontrado na literatura a descrição de polifosfatos e seu papel na embriogênese.

O aracnídeo *R. microplus* possui o desenvolvimento embrionário similar aos insetos. A confirmação destas similaridades é mais evidente quando se compara com a embriogênese do principal modelo no estudo da Biologia do Desenvolvimento, a mosca *Drosophila melanogaster* (Bate e Arias, 1991; Monnerat et al., 2002). No 4º dia de desenvolvimento do embrião em *R. microplus* inicia-se a formação de um blastoderma sincicial, e em seguida, o embrião se torna um organismo multicelular e começa a organogênese (Figura 1 do Anexo I, Campos et al., 2006). Estes dados possibilitam uma associação do metabolismo celular com os estágios de desenvolvimento em que o embrião se encontra. Esta abordagem permite que se defina em que evento durante o desenvolvimento o embrião mais necessita de um determinado metabólito, como é o caso do polifosfato.

A função do polifosfato como reserva de  $P_i$  é bem caracterizada em procariontes e também em eucariontes inferiores (Kulaev, 1975; Kulaev e Vagabov, 1983; Kornberg, 1995). Em *R. microplus*, o declínio do conteúdo de polifosfato total após o 7º dia de desenvolvimento não reflete o aumento do ortofosfato, que ocorre somente a partir do 12º dia, sugerindo que estes biopolímeros também possuem outras funções, além da de reserva para o desenvolvimento do embrião (Figura 8). Neste caso, uma fonte alternativa de  $P_i$  para o embrião poderia ser a defosforilação da vitelina, a principal proteína do ovo, que é gradualmente defosforilada durante a embriogênese (Silveira et al., 2006). Esta hipótese foi corroborada quando a atividade da exopolifosfatase foi

analisada em homogenato total. Foi verificado que a mesma manteve-se elevada após o 9º dia e o conteúdo de polifosfato total se mantém estável, onde o esperado seria que ele continuasse decaindo, indicando que o embrião está recebendo P<sub>i</sub> de outra fonte, a qual possibilita que os níveis de polifosfato total se mantenham estáveis nesta fase do desenvolvimento (Figura 9). O decréscimo do tamanho da cadeia do polifosfato foi confirmado comparando o tamanho das cadeias por eletroforese entre diferentes dias da embriogênese, e o mesmo foi refletido na atividade exopolifosfatásica em homogenato total (Figura 10).

Estudos mostram que além dos polifosfatos serem importantes como reserva de P<sub>i</sub>, nos eucariontes eles também participam em processos regulatórios (Kornberg et al., 1999). O polifosfato em diferentes compartimentos celulares tem sido mais estudado em *S. cerevisiae*, e o seu metabolismo depende da idade da cultura e das condições de cultivo (Kulaev, 1975; Kulaev e Kulakovskaya, 2000; Lichko et al., 2003). A participação do polifosfato nuclear em diversas etapas que ocorrem ao longo da via do gene à proteína é uma das mais importantes funções desses compostos em microorganismos eucariontes e procariontes, no qual tem sido bem documentada sua relevância no controle da expressão gênica, como por exemplo na adaptação à fase estacionária ou outras transições de desenvolvimento (Brown e Kornberg, 2004). Os nossos resultados confirmam que embriões de *R. microplus* possuem polifosfato no núcleo e que o conteúdo nuclear reflete a atividade da exopolifosfatase durante a embriogênese (Figura 11A). Esta mobilização é completamente diferente da mitocondrial (Figura 11B), e o conteúdo de polifosfato nuclear diminuiu no mesmo estágio de desenvolvimento em que o RNA total aumentou, após a formação do blastoderma celular no 5º dia de desenvolvimento (Figura 4C do Anexo I, Campos et al., 2006).

Tem sido demonstrado que o polifosfato está envolvido em vários aspectos da transcrição do RNA sendo capaz de interagir fisicamente com a RNA polimerase (Kusano e Ishihama, 1997). Também há relatos sobre o envolvimento destas moléculas na estabilidade do mRNA (Blum et al., 1997), no aumento da produção de proteínas (Itoh et al., 2006) e no aumento da estabilidade de polissomos *in vivo* durante a síntese protética (McInerney et al., 2006). Em núcleo de fígado de rato, o polifosfato foi relacionado com a fração

protéica não correspondente às histonas, podendo interagir com o complexo DNA-histona se ligando à cromatina, e esta ligação possibilita a inibição da atividade de algumas enzimas nucleares, incluindo a topoisomerase (Schroder et al., 1999). Todos estes dados dão suporte a hipótese de que os polifosfatos estão envolvidos na regulação gênica em eucariontes superiores. A simultânea mobilização e ativação de polifosfatos nucleares e de RNA total (Campos et al., 2008) durante a embriogênese do *R. microplus* sugere uma participação destes compostos na regulação gênica. Esta hipótese foi analisada quando somente a atividade da exopolifosfatase nuclear foi estimulada por RNA total, em contraste com a mitocondrial (Figura 12). Este dado sugere que, além da possível função dos polifosfatos na regulação gênica como relatado na literatura, o ambiente nuclear é também responsável pela regulação da atividade exopolifosfatásica.

Para o desenvolvimento do embrião, além de fatores regulatórios como os RNAs, outros constituintes necessários ao embrião são fornecidos na ovogênese. Durante a ovogênese, o ovário e os ovócitos de artrópodes crescem rapidamente, acumulando grandes quantidades de carboidratos, lipídeos e proteínas. A quantificação desses constituintes durante a embriogênese do *R. microplus* sugere que os lipídeos e os carboidratos são as principais fontes de energia para o embrião, principalmente durante a fase inicial da embriogênese, onde os lipídeos totais apresentam uma diminuição em torno de 45% entre o 5º e 7º dia do desenvolvimento embrionário, e os carboidratos totais reduzem 30% entre o 7º e o 9º dia (Figuras 4A e 4B do Anexo I, Campos et al., 2006). Os lipídeos estariam sendo utilizados na primeira parte da embriogênese, servindo para o suporte energético durante a celularização, como também foi observado em *D. melanogaster* (Bate e Arias, 1991). Nesta condição, este processo está sendo direcionado pelo organismo materno, porque até o início da diferenciação celular todo o controle ainda está sendo dirigido por genes exógenos ao embrião (Bate e Arias, 1991). Por outro lado, os carboidratos seriam as principais reservas energéticas para a rápida segmentação do embrião, um processo de natureza zigótica, marcando o início do controle metabólico e gênico das próprias células do embrião (Nusslein-Volhard e Roth, 1989; Bate e Arias, 1991).

As proteínas são os principais componentes do vitelo de ovos de artrópodes, sendo a vitelina a sua principal constituinte. O conteúdo de proteína total dos ovos do *R. microplus* permanece inalterado durante toda a embriogênese (Figura 5 do Anexo I, Campos et al., 2006), o que está de acordo com os mesmos dados obtidos de outro carrapato, o *Hyalomma dromedarii* (Kamel e Fahmy, 1982). Por outro lado, resultados de nosso grupo mostram que ocorre um decréscimo do conteúdo de vitelina durante os primeiros quatro dias da embriogênese (Figura 5 do Anexo I, Campos et al., 2006, Logullo et al., 2002), momento este em que não houve detecção de guanina (Moraes et al., 2007). A guanina é classicamente descrita como o único produto de degradação de aminoácidos em aracnídeos (Urich, 1990). Essa diminuição do conteúdo de vitelina sem uma queda paralela das proteínas totais e sem o surgimento de guanina, na primeira fase da embriogênese, sugere que os aminoácidos da vitelina poderiam estar sendo mobilizados apenas para a construção de novas proteínas necessárias para o desenvolvimento do embrião. Este fenômeno é esperado, principalmente nos quatro primeiros dias da embriogênese, onde os aminoácidos derivados da quebra da vitelina provavelmente darão suporte à formação do blastoderma celular. A rápida cinética de desenvolvimento embrionário do *R. microplus* requer um grande suporte energético. De fato, um aumento do consumo de oxigênio dos ovos foi visto até o 12º dia de desenvolvimento, período que inclui a celularização e segmentação do embrião (Figura 2 do Anexo I, Campos et al., 2006). Este dado confirma a importância do metabolismo mitocondrial para o desenvolvimento embrionário deste carrapato.

As funções dos polifosfatos na mitocôndria estão associadas com a fosforilação oxidativa e com a síntese de ATP (Beauvoit et al., 1989). Desacopladores inibiram a acumulação de polifosfato em mitocôndrias de *S. cerevisiae* e após “sonicação” ocorreu uma rápida hidrólise dos polifosfatos sugerindo que a acumulação de polifosfatos depende da força próton – motriz da membrana (Pestov et al., 2004; Pestov et al., 2005). Foi demonstrado que a exopolifosfatase mitocondrial de *R. microplus* é estimulada em 100% por NADH, 35 % por ADP e é completamente inibida por P<sub>i</sub> (Figuras 13A, 13B e 13C) sugerindo uma regulação por P<sub>i</sub> e por carga energética. Esta atividade

exopolifosfatásica aumentou durante a respiração mitocondrial quando piruvato ou succinato foi adicionado, e o efeito estimulatório desapareceu após a adição de KCN, um inibidor da fosforilação oxidativa (Figura 14A). Estes resultados indicam que o gradiente de prótons e/ou o fluxo de elétrons possivelmente regulam a exopolifosfatase para fornecer  $P_i$  para a síntese de ATP. Esta hipótese foi confirmada, ao medir o consumo de  $O_2$  mitocondrial dependente de ADP na presença de polifosfato e na ausência de qualquer outra fonte de  $P_i$  (Figura 14B).

Após o isolamento da fração solúvel e de membrana mitocondrial foi verificado que a maior atividade exopolifosfatásica se encontra na fração de membrana (Tabela 5). Este dado sugere que é a exopolifosfatase de membrana a responsável por fornecer  $P_i$  do polifosfato para a síntese de ATP, já que foi possível inibir completamente o consumo de  $O_2$  ADP-dependente utilizando heparina (Figura 15A). Para analisar um possível papel da exopolifosfatase solúvel são necessários estudos sobre sua regulação e função. Na literatura já foi relatada a regulação de cinases por polifosfatos (Smith et al., 2006), portanto uma hipótese para a sua função seria a regulação de cinases mitocondriais, já que foi verificado anteriormente que as mesmas possuem papel importante na defesa antioxidante, prevenindo a formação de espécies reativas de oxigênio (ROS) na mitocôndria (da-Silva et al., 2004; Meyer et al., 2006). Nossos resultados indicam que os polifosfatos provavelmente estão sendo mobilizados em uma situação de grande necessidade energética, o que resultaria em um aumento na formação de ROS.

Para analisar um possível papel fisiológico do polifosfato mitocondrial, os seus níveis foram quantificados e a atividade da exopolifosfatase mitocondrial foi medida ao longo da embriogênese. A maior atividade exopolifosfatásica associada com o maior consumo dos polifosfatos ocorreu durante a formação do blastoderma celular e segmentação do embrião, entre o 5º e 7º dia de desenvolvimento (Figura 11B). Dessa maneira, a atividade da exopolifosfatase mitocondrial parece estar correlacionada com a demanda energética do embrião durante esta fase do desenvolvimento embrionário, na qual o embrião utiliza grande parte de suas reservas de carboidratos e lipídeos (Figuras 4A e 4B do Anexo I, Campos et al., 2006). Após o 7º dia de desenvolvimento, o

conteúdo de polifosfato mitocondrial aumenta, enquanto a atividade exopolifosfatásica diminui, ficando estável entre o 12º e 18º dia de desenvolvimento. Estes resultados sugerem que no final da embriogênese ocorre uma mudança no balanço da síntese e hidrólise de polifosfatos mitocondriais. Neste caso, uma fonte alternativa de P<sub>i</sub> poderia ser o polifosfato citoplasmático ou mesmo o P<sub>i</sub> proveniente da defosforilação da vitelina (Silveira et al., 2006).

Os parâmetros cinéticos das duas exopolifosfatas, nuclear e mitocondrial, foram comparados. Exopolifosfatas descritas em procariontes apresentam maior afinidade para hidrólise de polifosfatos de cadeia longa (Kumble e Kornberg, 1996) e o mesmo é observado para microorganismos eucariontes, porém algumas enzimas de *S. cerevisiae* e *Leishmania major* são mais ativas, hidrolisando polifosfatos de cadeia curta, como o poly P<sub>3</sub> (Kumble e Kornberg, 1996; Rodrigues et al., 2002). Em *R. microplus*, os resultados mostraram que a afinidade da enzima nuclear praticamente não se alterou com os substratos testados, porém, a mitocondrial apresentou um K<sub>m</sub> 10 vezes menor para o poly P<sub>3</sub> quando comparado com o poly P<sub>15</sub> e o poly P<sub>65</sub> (Tabela 4). Estes resultados indicam que a exopolifosfatase nuclear é única, pois em nosso conhecimento não há relato na literatura de uma enzima que atua com praticamente a mesma afinidade entre polifosfatos de cadeias curtas e longas, e a exopolifosfatase mitocondrial faz parte de um grupo específico que apresenta maior afinidade para polifosfatos de cadeias curtas.

A Exopolifosfatase de *E. coli*, a qual pertence à superfamília kinase/actina/hsp-70, requer cátions divalentes e K<sup>+</sup> para sua atividade máxima, enquanto a de levedura, que pertence à superfamília da DHH fosfoesterases, requer somente cátions divalentes (Lichko et al., 2003). A exopolifosfatase nuclear de *R. microplus* foi estimulada por Mg<sup>+2</sup> e Co<sup>+2</sup> enquanto a mitocondrial foi estimulada somente por Mg<sup>+2</sup> (Figura 16A), e ambas foram insensíveis a K<sup>+</sup>, indicando que as exopolifosfatas destas frações celulares de *R. microplus* são parecidas com a superfamília da DHH fosfoesterases.

São poucos os compostos descritos como efetivos inibidores da atividade exopolifosfatásica (Kornberg et al., 1999). Tratamentos com molibdato, um inibidor de fosfohidrolases, e fluoreto, um inibidor de

pirofosfatases, não inibiram nenhuma das duas exopolifosfatases de *R. microplus* testadas (resultado não mostrado). Entretanto, a heparina, um bom inibidor para exopolifosfatase de bactérias e leveduras (Lichko et al., 2003), foi efetiva em ambas as frações, sendo a cinética de inibição diferente, onde a mitocondrial foi mais sensível a heparina do que a nuclear, embora as duas fossem quase totalmente inibidas (Figura 16B). Tais diferenças cinéticas confirmam a existência de duas diferentes enzimas expressas em núcleo e mitocôndrias, e refletem as diferentes funções dos polifosfatos nestes compartimentos celulares para o desenvolvimento do embrião.

## CONCLUSÕES

## 6 Conclusões

- 1) Em geral, as variações do conteúdo de polifosfato acompanharam a variação da atividade exopolifosfatásica, indicando a importância destas enzimas no controle do metabolismo de polifosfatos na embriogênese do *R. microplus*.
- 2) Polifosfatos possuem outras funções durante o desenvolvimento embrionário do *R. microplus*, não sendo exclusivamente uma fonte de reserva de fosfato.
- 3) O corpo de dados sugere a presença de duas distintas formas de esopolifosfatas, uma presente na mitocôndria e outra no núcleo, todavia os resultados sugerem que ambas são parecidas com a superfamília da DHH fosfoesterases.
- 4) A exopolifosfatase nuclear é ativada por RNA total, e este dado fornece uma nova perspectiva sobre o fenômeno previamente descrito da regulação gênica mediada por polifosfato.
- 5) A exopolifosfatase mitocondrial é regulada por demanda de fosfato e parcialmente por carga energética.
- 6) O polifosfato induz um consumo de oxigênio ADP-dependente, funcionando como fonte de fosfato para a síntese de ATP.
- 7) Fisiologicamente, o polifosfato mitocondrial é consumido especificamente nos estágios da embriogênese em que o embrião utiliza as principais reservas energéticas do ovo, sugerindo que o polifosfato mitocondrial esteja envolvido com a necessidade energética do embrião.
- 8) Foram identificadas atividades exopolifosfatásicas associadas tanto às frações de membranas mitocondriais quanto às frações solúveis. E pelo menos a fração de membrana está possivelmente envolvida com o processo de síntese de ATP por apresentar um consumo de oxigênio ADP-dependente .

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Eldo Campos, 2008

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Veterinary Parasitology 138 (2006) 349–357

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## Kinetics of energy source utilization in *Boophilus microplus* (Canestrini, 1887) (Acari: Ixodidae) embryonic development

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Received 16 June 2005; received in revised form 23 January 2006; accepted 5 February 2006

### Abstract

The present work evaluates the kinetics of utilization of the main potential energy sources throughout the embryonic developmental stages of *Boophilus microplus*. The embryonic development of this arthropod is completed in 21 days. Cellularization of the blastoderm occurs on the 6th day and is rapidly followed by germ band extension and segmentation, whose first signs are visible on the 7th day. Cellularization is typically a maternal-driven process, carried out by molecular determinants deposited in the oocyte during oogenesis. On the other hand, segmentation is of zygotic nature, being the consequence of the synthesis of various components by the growing embryo. The enhancement in total *B. microplus* RNA was observed after cellularization, corroborating the replacement of maternal-driven processes by embryonic zygotic expression. An abrupt increase in oxygen consumption was observed from cellularization until the 8th day of development. The reduction in dry weight at the same period and the susceptibility of oxygen consumption to KCN suggest that the respiration process is activated during early embryonic development. A marked decrease in total lipid content occurred between the 5th and 7th days of development, suggesting this is the main energy source for cellularization. A major reduction in carbohydrate content occurred later, between the 7th and 9th days, and it could be assigned to the morphological segmentation of the embryo. Although the total amount of proteins remains unchanged from oviposition to hatching, a 15% reduction in vitellin (VT) content was observed

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before cellularization, up to the 4th day after egglaying. This observation was correlated to the synthesis of new proteins needed to support early embryo development. Additional 20% of VT was consumed thereafter, mainly at the end of embryogenesis, and in this case VT is probably used as energy source to the older embryo. Altogether, these data indicate different energy sources for maternal and zygotic driven processes.

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**Keywords:** Egg; Energy metabolism; *Boophilus*; Embryogenesis; Yolk proteins

## 1. Introduction

Bovine cattle blood is the unique feeding source supporting development of the Ixodidae tick *Boophilus microplus*. This ectoparasite is also the vector of babesiosis and anaplasmosis, imposing serious economic losses to several countries (Guerrero et al., 2005). Larval, nymphal, and adult development of this tick takes place on a single bovine host, within a period of 2–4 weeks. The adult female, after completion of engorgement, drops off the host and initiates oviposition 3 days later. Hatching occurs around 21 days after egglaying and the resulting larvae can survive many months before finding a new host, where development proceeds up to adult stages (Corson et al., 2004).

This arthropod increases its body weight about 100 times after blood meal. As a consequence of digestion, a great amount of food is processed and used in yolk synthesis, wherewith the growing embryo meets energy and nutrition needs (Sonenshine, 1991). The main yolk protein, called Vitellin (VT), is a heme-protein in ticks and is responsible for the brown egg color (Boctor and Kamel, 1976; Rosell and Coons, 1991; James and Oliver, 1997; Logullo et al., 2002). Heme-proteins are involved in a wide variety of biological processes, including respiration, oxygen transport and oxygen metabolism (Ponka, 1997). Oxygen consumption is a major parameter characterizing respiratory metabolism, and has been widely used to study the influence of several environmental factors in arthropod development and physiology (Madhavan, 1975; Braune, 1976; Tonsetich et al., 1976; Petitpren and Knight, 1979).

As a rule, oviparous animals face embryogenesis in the absence of exogenous nutrients. In this case, maternal nutrients are packaged into the oocytes, stored mostly as yolk granules (Fagotto, 1990; Sappington and Raikhel, 1998). Maturation occurs during the pre-oviposition phase, characterized by a rapid increase in ovarian size (Cherry, 1973). It is at this phase that

oocytes exhibit fast growth, accumulating RNAs, carbohydrates, lipids and proteins that will meet the regulatory and metabolic needs of the developing embryo (Chippendale, 1978). Lack of any required yolk component may restrain or even block embryo development inside the egg (Thompson and Russel, 1999). Previous studies performed with birds have focused on the importance of various egg components to the developing embryo, as well as on the mechanisms that transport nutrients from the yolk to their utilization sites in embryonic tissues (Speake et al., 1998).

Current literature provides ample data regarding the regulatory cascade of events that control development during arthropod embryogenesis. Nevertheless, little information is available concerning energy metabolism during embryonic development. Knowledge of this process could reveal potential targets to control arthropod disease vectors or agricultural pests. The *B. microplus* tick is an ectoparasite responsible for weight losses and skin damage in bovine cattle. Apart from this, the organism is a vector of several diseases.

Here we quantified the major classes of potential energy sources in the course of *B. microplus* embryogenesis. Oxygen consumption was also measured in order to follow the respiratory metabolism. These data were correlated to the different embryonic stages, whose morphology was evaluated by confocal microscopy. We suggest that distinct sources of energy are mobilized in the course of *B. microplus* embryogenesis, and that switches among different energy sources occur at specific development stages.

## 2. Materials and methods

### 2.1. Ticks

Ticks were obtained from a colony maintained at the Faculdade de Veterinária, Universidade Federal do

Rio Grande do Sul, Brazil. *B. microplus* ticks from the Porto Alegre strain, free of *Babesia* spp., were reared on calves obtained from a tick-free area. Engorged adult females were kept in Petri dishes at 28 °C and 80% relative humidity upon completion of oviposition, which starts about 3 days after dropping off the calves. Eggs were collected on different days during embryogenesis and stored at –20 °C.

## 2.2. Histological procedures

*B. microplus* eggs underwent the protocol classically used to dechorionate *Drosophila melanogaster* embryos (Forbes and Ingham, 1993). Briefly, eggs were submerged in a 20% sodium hypochlorite bath for 2–4 min. After rinsing in water, eggs were fixed in a biphasic 10:9:1 mixture of heptane: 4% paraformaldehyde in PBS: dimethyl sulfoxide (DMSO), for 20 min under mild agitation. The lower aqueous phase (containing the fixative plus DMSO) was then replaced with 100% methanol and the remaining biphasic mixture was vigorously shaken for 30 s. Completely or partially dechorionated *B. microplus* eggs fell to the bottom of the tube and were collected, rinsed twice with 100% methanol and stored at –20 °C. Prior to use, eggs were submitted to a serial rehydration with 70, 50 and 30% ethanol in PBS, followed by three washes in PBS. Incubation with Acridine Orange or Evans Blue (both at 0.01% in PBS) was performed for 20 min and enabled the visualization of nuclei and cell limits, respectively. Whole eggs were directly observed on a Zeiss LSM 410 laser scanning confocal microscope, using a 488 nm laser beam and a BP 510–525 nm filter for the visualization of acridine orange or, alternatively, a 543 nm laser beam and a LP 570 nm filter for Evans blue, respectively.

## 2.3. Oxygen consumption

The total oxygen consumed by embryonated eggs at different times after oviposition was assayed using a Clark-type electrode (YSI, mod. 5775, Yellow Springs, OH). The calibration process was executed using 100% as complete air-saturated buffer at 28 °C. Measurements were carried out in 3 mL 20 mM PBS buffer (pH 7.4); 0.1 mM EDTA, and the rate of oxygen consumption was calculated in  $\mu\text{mol O}_2/\text{min mg egg}$ . A solution containing 1 mM KCN was added to inhibit

cytochrome oxidase. The same solution was used as negative control. For each day after oviposition evaluated, three assays were performed using 200 mg of eggs.

## 2.4. Determination of dry weight and water content

Three samples of 300 eggs were used in each determination. After counting, groups of eggs at different days after oviposition were weighed on a Shimadzu balance AX200 and dried at 85 °C until constant weight was attained. The water content was calculated as the difference in weight values before and after drying. Three samples were analyzed for each experimental point.

## 2.5. RNA content

RNA was extracted from 50 mg of egg homogenates with Tri-reagent (Sigma, inc.) according to the manufacturer's instructions and precipitated with 0.5 mL isopropanol for 10 min at room temperature. Subsequently, the extracted RNA was washed with 1 mL 75% ethanol, vacuum-dried for 10 min and resuspended in 1 mL water. Absorbance was measured at 260 and 280 nm, assuming that one absorbance unity at  $A_{260}$  corresponds to 40  $\mu\text{g}$  RNA (Sambrook et al., 1989).

## 2.6. Total protein content

Five milligrams of eggs were homogenized in 1 mL PBS 20 mM pH 7.4, leupeptin 100  $\mu\text{M}$ , pepstatin 100 nM and benzamidine 1 mM and centrifuged at 11,000  $\times g$  for 5 min. Total protein content was quantified according to Bradford (1976) in 10  $\mu\text{L}$  aliquots, using bovine serum albumin as standard. Three samples were analyzed for each experimental point.

## 2.7. Total sugar content

Ten milligrams of eggs were homogenized in 1 mL PBS 20 mM pH 7.4 and centrifuged at 11,000  $\times g$  for 5 min. Aliquots of 50  $\mu\text{L}$  were added to 20  $\mu\text{L}$  80% phenol, 1 mL  $\text{H}_2\text{SO}_4$  and 350  $\mu\text{L}$  water, vortexed and then left at room temperature for 30 min. Absorbance was read at 490 nm using a molar extinction coefficient of  $6.22 \text{ M}^{-1}$  (Dubois et al., 1956), and

total sugar content was calculated based on a glucose standard curve. Three samples were analyzed for each experimental point.

#### 2.8. Total lipid content

Egg homogenate aliquots containing 10 mg of protein each were extracted with chloroform–methanol–water (2:1:0.5; v/v) (Bligh and Dyer, 1959). The organic phase was dried under a N<sub>2</sub> stream and weighed to determine lipid content. Three samples were analyzed for each experimental point.

#### 2.9. Vitellin quantification

Determination of VT content was performed using an enzyme-linked immunosorbent assay (ELISA). Egg homogenates from the 1st to the 20th day of embryogenesis (90 ng protein) in PBS were spotted onto ELISA plates as 100 µL aliquots and incubated overnight. Plates were blocked with 5% (W/V) non-fat dry milk, 0.5% (V/V) Tween 20 in PBS, and incubated with anti-VT polyclonal antibody (Logullo et al.,

2002) diluted 1:5000 in PBS containing 5% non-fat dry milk for 2 h. Goat anti-rabbit IgG conjugated to Alkaline Phosphatase (Sigma, inc.) diluted to 1:10,000 (V/V) was used as secondary antibody. After 90 min incubation in *p*-nitrophenyl phosphate, which served as substrate, the amount of VT in each sample was determined using a Thermomax microplate reader (Molecular Devices, Sunnyvale CA). A standard curve was made using VT purified from eggs collected on the 1st day after oviposition.

### 3. Results

The present work correlates the mobilization kinetics of some components of *B. microplus* eggs with the morphological changes that take place during early embryogenesis.

#### 3.1. Morphology

The early stages of *B. microplus* embryogenesis were visualized by confocal scanning microscopy

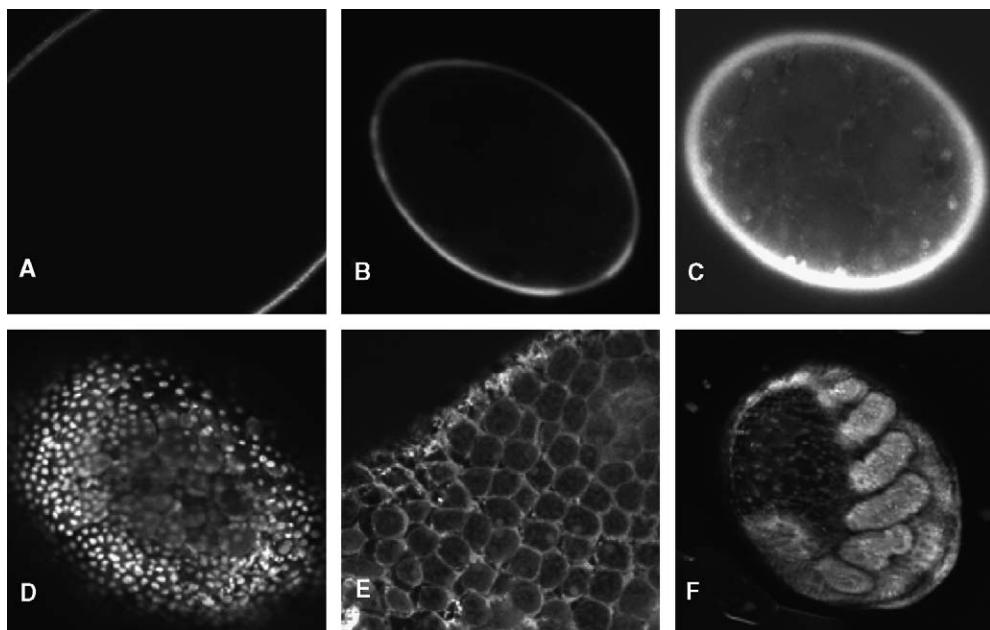


Fig. 1. Morphology of early *B. microplus* embryos. Permeabilized eggs of different days after oviposition were submitted to laser scanning confocal microscopy. (A) No labeling (only auto-fluorescence); (B–F) Acridine Orange labeling; (E) Evans blue labeling. (A, D, E) 6-day-old; (B) 1-day-old; (C) 4-day-old; (F) 7-day-old embryo. A black and white projection of a 3D reconstruction of a well-segmented embryo is shown in panel F. Note that only the chorion is auto-fluorescent (panel A). Acridine Orange and Evans blue labeling show nuclei and cell limits, respectively.

(Fig. 1). It was first verified that, with exception of the chorion, embryos of this species are not auto-fluorescent (Fig. 1A), a characteristic that allowed labeling with exogenous markers. Visualization of nuclei labeled with Acridine Orange revealed almost no staining on the 1st day after oviposition (Fig. 1B). On the 4th day, nuclei had proliferated and attained the cortical cytoplasm, a pattern typical of syncytial blastoderm (Fig. 1C). A mitotic burst occurred between days four and six, as judged by the density of nuclei observed on the 6th day after oviposition (Fig. 1D). Evans Blue staining of 6-day-old embryos, but not of earlier ones, confirmed that cellularization had taken place (Fig. 1E), characterizing the cellular blastoderm stage. Afterwards, embryo development quickly progressed to the germ band extension phase: on the 7th day after egg laying, the embryo was clearly distinguished from the extra-embryonic membrane and segmentation was evident (Fig. 1F).

### 3.2. Oxygen consumption

No detectable oxygen consumption was verified on the 1st day after oviposition. From that point on, intense activation of the respiration process occurred until the 12th day of embryonic development. Oxygen consumption was completely blocked at all stages by addition of 1 mM KCN in the reaction medium (Fig. 2).

### 3.3. Determination of dry weight and water content

Both the *B. microplus* eggs' dry weight and the water content remained constant during early development, up to the 6th day after egg laying (Fig. 3). A decrease in dry weight was observed between the 7th and 9th days, when the total dry mass dropped from  $15.7 \pm 1.3$  to  $12.6 \pm 1.4$  µg/egg, corresponding to a loss of 18.4%. At the same time, the water content increased from  $24.3 \pm 1.3$  to  $27.2 \pm 1.4$  µg/egg of the total eggs' weight, corresponding to 11.2% (Fig. 3).

### 3.4. Consumption of energetic sources during embryogenesis

The total lipid content remained stable until the 5th day ( $2.07 \pm 0.46$  µg/egg), dropping to approximately 1.1 µg/egg on the 7th day, a value that was kept roughly

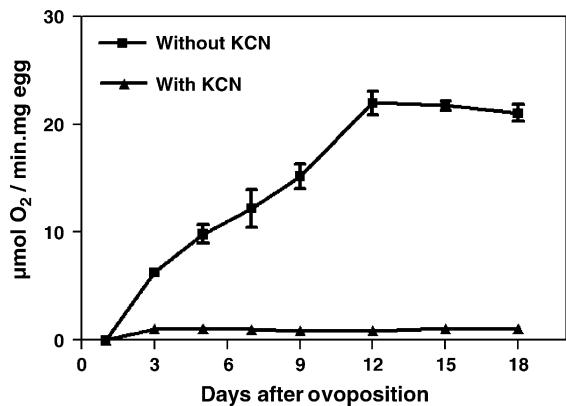


Fig. 2. Oxygen consumption. Oxygen consumption of *B. microplus* embryos was assayed using a Clark-type electrode calibrated to 100% with air-saturated buffer at 28 °C. (■) Kinetics of oxygen consumption at different days after oviposition. (▲) effect of a 1 mM KCN solution, inhibitor of cytochrome oxidase. Aliquots containing 200 mg of eggs were assayed in all cases. Data shown are mean  $\pm$  S.E.M. ( $n = 3$ ).

unchanged until hatching (Fig. 4A). Total sugars exhibited similar kinetics, although slightly delayed: values remained stable until the 7th day ( $1.86 \pm 0.40$  µg/egg), dropped to  $1.18 \mu\text{g}/\text{egg}$  on the 9th day (a 36.6% decrease) and were kept constant until hatching (Fig. 4B). The sugar and the lipid kinetics accompanied the dry weight changes, shown in Fig. 3. The changes in total RNA amount during embryogenesis are shown in Fig. 4C. The total amount of RNA was almost constant during the first 3 days, rose quickly between the 6th and the 9th days after oviposition, and did not exhibit significant changes thereafter.

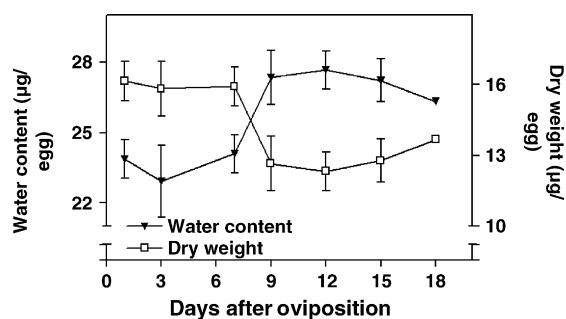


Fig. 3. Changes in dry weight and water content. The dry weight of eggs at different days after oviposition was determined with an analytical balance to the nearest 0.0001 g. Each aliquot contained 300 eggs. The water content was calculated as the difference in weight values before and after drying. Data shown are mean  $\pm$  S.E.M. ( $n = 3$ ).

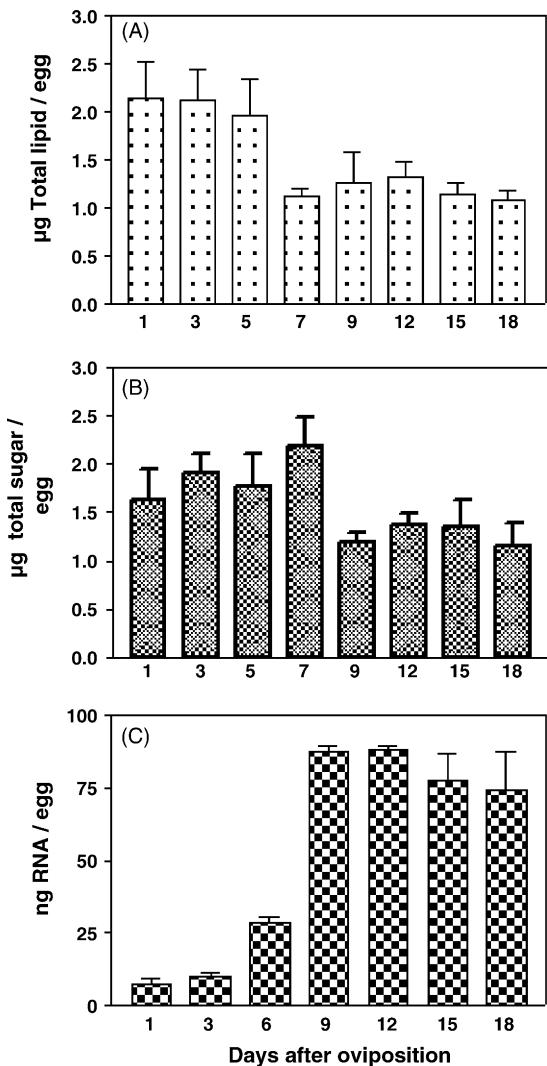


Fig. 4. Consumption of energetic sources. Major *B. microplus* egg storage components were quantified at different days after oviposition. (A) Lipid quantification, determined by gravimetric method (Bligh and Dyer, 1959); (B) total sugar concentration, measured using Dubois' method; (C) total RNA, extracted from egg (50 mg) homogenate; absorbance was read at 260 and 280 nm, using a molar extinction coefficient of  $6.22 \text{ M}^{-1}$ . Data shown are mean  $\pm$  S.E.M. ( $n = 3$  or 4).

### 3.5. Protein mobilization

Vitellin (VT), the main yolk protein, represents 80% of total *B. microplus* egg protein content on the 1st day after oviposition. No significant changes in total protein content were observed throughout *B. microplus*

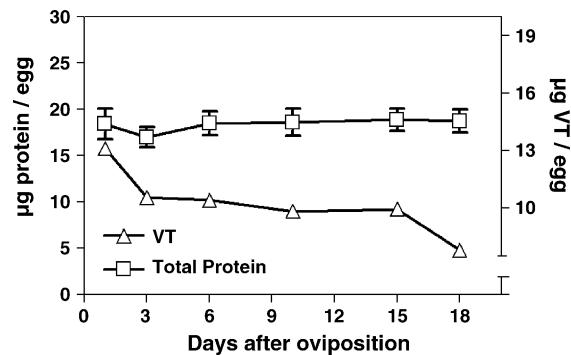


Fig. 5. Protein mobilization. Total protein concentration was measured, during *B. microplus* embryogenesis, using Bradford's method. The amount of VT in egg homogenates was determined using anti-VT polyclonal antibody with the enzyme-linked immunosorbent assay (ELISA). Data shown are mean  $\pm$  S.E.M. ( $n = 3$  or 4).

embryogenesis (Fig. 5). In contrast, around 15% of VT is mobilized during the initial 4 days of embryonic development while an additional 20% of VT is consumed until the 18th day after oviposition (Fig. 5).

### 4. Discussion

Development in invertebrates has been analyzed under different aspects, morphogenesis and cell cycle control being outstanding examples (Nusslein-Volhard and Roth, 1989; Bate and Arias, 1993; Edgar et al., 1994; Patel, 1994). Mobilization of yolk components, especially Vitellin, the major protein constituent of arthropod's eggs, has also been the subject of several reports (Takahashi et al., 1993; Abreu et al., 2004). However, there is a lack of information regarding the dynamics of energetic metabolism during embryogenesis.

The metabolic costs of embryonic development can be defined as the total amount of energy consumed by an embryo during its development, including the demands by growth or biosynthesis and by tissue maintenance (Thompson and Stewart, 1997). The present work evaluated the kinetics from the utilization of the main potential energy sources available during the embryonic development of the cattle tick, *B. microplus*. As information on tick embryo development was but scarcely available (Sonenshine, 1991), we first evaluated the morphology of embryos mainly on the early phases of their development.

The *B. microplus* embryonic development is completed in 21 days when the specimens are reared at 28 °C. Until the 5th day after oviposition, the *B. microplus* embryo is a syncitium. Cellularization of the blastoderm occurs on the 6th day, being followed by impressively quick movements: by the 7th day a segmented germ band can already be distinguished (Fig. 1). Rapid early embryonic movements are typical of the model system *D. melanogaster* and have also been observed in mosquitoes (Bate and Arias, 1993; Monnerat et al., 2002).

Cellularization of *D. melanogaster* embryos is a synchronous process and a maternal-driven phenomenon, regulated exclusively by factors deposited in the oocyte during oogenesis. In this species, cellularization is a key event that definitively switches control of the embryonic development to the embryo itself: cells loose synchrony and the progression to later stages relies on zygotic transcription (Bate and Arias, 1993).

The quick developmental kinetics observed in early *B. microplus* embryos requires a readily available energetic support. In fact, an increase in oxygen consumption was noted until the 12th day after oviposition, a period that includes the cellularization and segmentation of *B. microplus* embryos. Oxygen consumption was completely inhibited by addition of KCN, further confirming the activation of the respiration process (Fig. 2). To our knowledge, evaluations of oxygen consumption during the life cycle of arthropods are restricted to post-embryonic development stages (Guerra and Cochran, 1970; Petitpren and Knight, 1979; Gromysz-Kalkowska and Lechowski, 1992). Parallel to the abrupt rise in oxygen consumption at the beginning of development, a significant reduction of *B. microplus* eggs' dry weight is observed, corroborating an active respiration process. Moreover, this is accompanied by an increase in egg total water content, detectable between the 7th and the 9th days after oviposition (Fig. 3). Increase in water content is probably a consequence of the intense metabolic activity and respiration that take place inside the egg during this period (Figs. 2 and 3). It is noteworthy that a decrease in *B. microplus* eggs' dry weight occurs mainly after the tick embryo cellularization, when segmentation is rapidly achieved. The reduction in dry weight and the increase in oxygen consumption were also observed in the fish *Solea senegalensis*, but only in the latest embryonic stages (Parra et al., 1999).

Quantification of the major constituents of the egg in the course of *B. microplus* embryogenesis suggests that lipids and carbohydrates are the major energy sources, specifically during early embryo development. For both classes of molecules, amounts were observed to remain the same, except between the 5th and the 7th days for lipids and between the 7th and the 9th days for carbohydrates. This kinetics suggests the utilization of lipids in the course of cellularization, a maternal-driven process (Bate and Arias, 1993). On the other hand, carbohydrates would be the major energy source for the quick segmentation of the embryo, of zygotic nature (Nusslein-Volhard and Roth, 1989; Bate and Arias, 1993).

Changes in nucleic acids content, particularly RNA, reflect the anabolic activities of the developing arthropod embryo and the degree of embryonic independence of the material of maternal origin. The increase in *B. microplus* total RNA content between the 6th and the 9th days after oviposition confirms an intense zygotic transcription activity after cellularization of the blastoderm, when development proceeds towards segmentation. These data suggest that maternal-driven processes are replaced by embryonic zygotic expression after cellularization of the blastoderm. A steady increase in total RNA content at the beginning of embryonic development has also been described for *Tribolium confusum* and for the tick *Hyalomma dromedarii* (Amnai-Devi et al., 1963; Kamel et al., 1982).

Proteins are the largest components of the yolk and VT is its major constituent. The total protein content in *B. microplus* eggs remains unchanged from oviposition to hatching (Fig. 5). This is in agreement with data from the tick *H. dromedarii* (Kamel et al., 1982). However, a significant decrease in *B. microplus* VT content during the first 4 days of embryogenesis was verified. An additional decrease in VT was observed between the 6th and the 18th days of development. The decrease in VT content without a parallel depletion of total proteins suggests that its final destination should be the synthesis of new proteins. This is expected to be the case, mainly with the VT depletion during the first 4 days, when VT-derived amino acids are probably the source of newly synthesized proteins that will support blastoderm formation. Guanine is the product of amino acid catabolism in arachnids (Urich, 1990) and its quantification in *B. microplus*' eggs revealed an increase in the

concentration of this metabolite after cellularization, on the 6th day (data not shown). Therefore, part of the VT that is consumed from that point on could provide the amino acids to be used as energy source to the growing embryo, at later development stages (Fig. 5).

It should be noted that only 40% of the VT deposited in *B. microplus* oocytes is utilized during embryogenesis (Logullo et al., 2002). It is essential for the newly hatched tick larvae to identify a new host in order to proceed to further development stages. Their survival depends upon the remaining nutritional supply brought from the egg and on the metabolic rate of energy consumption during the early stages of post-embryonic development (Sonenshine, 1991).

In conclusion, it is likely that the energetic metabolism of *B. microplus* embryos is a dynamic process which varies with the progression of morphological stages and with the control of the cell cycle during development. Early embryogenesis requires maternal regulatory factors, like specific RNAs, deposited in the oocyte during oogenesis. An intense translational activity is inferred from the depletion of the major yolk protein, VT, without concomitant decrease in the embryo total proteins. With cellularization of the blastoderm, on the 6th day, the embryo itself assumes the control of its development, through activation of zygotic expression. This fact is reflected in the increase of total RNA, starting on the 6th day after oviposition. The development progresses quickly to segmentation, whose first signs are already visible on the 7th day after egglaying. An intense metabolism during embryo cellularization and segmentation is reflected both on the decrease in total egg's dry weight and on the increase of oxygen consumption at this period. A switch in energy source is also proposed to occur in parallel to the progression from maternal to zygotic control of development, since our data suggest lipids as the main energy source during cellularization, with carbohydrates accomplishing this role during embryo segmentation.

*B. microplus* is a one-host tick that causes major losses to bovine herds, especially in tropical regions, and major efforts have been made to develop immunoprophylactic tools against it. In this sense, knowledge of the processes involved in embryonic development may unravel additional targets that could be effective in the control of this ectoparasite.

## Acknowledgements

We are grateful to Cristóvão Barros Pinheiro for his excellent technical assistance. The authors are indebted to Regina Aparecida Caldeira for a critical reading of the manuscript. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ, Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq and Programa de Núcleos de Excelência – PRONEX.

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## Exopolyphosphatases in nuclear and mitochondrial fractions during embryogenesis of the hard tick *Rhipicephalus (Boophilus) microplus*

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### ARTICLE INFO

#### Article history:

Received 15 May 2008

Received in revised form 22 July 2008

Accepted 22 July 2008

Available online 25 July 2008

#### Keywords:

Polyphosphate

Embryogenesis

Exopolyphosphatase

*Rhipicephalus boophilus microplus*

Arthropoda

Metabolism

### ABSTRACT

The present work evaluated polyphosphate (poly P) metabolism in nuclear and mitochondrial fractions during *Rhipicephalus microplus* embryogenesis. Nuclear poly P decreased and activity of exopolyphosphatase (PPX – polyphosphate-phosphohydrolases; EC 3.6.1.11) increased after embryo cellularization until the end of embryogenesis. The utilization of mitochondrial poly P content occurred between embryo cellularization and segmentation stages. Increasing amounts of total RNA extracted from eggs progressively enhanced nuclear PPX activity, whereas it exerted no effect on mitochondrial PPX activity. The decline in total poly P content after the 7th day of embryogenesis does not reflect the free  $P_i$  increase and the total poly P chain length decrease after embryo cellularization. The  $K_{m,app}$  utilizing poly  $P_3$ , poly  $P_{15}$  and poly  $P_{65}$  as substrate was almost the same for the nuclear fraction (around 1  $\mu\text{M}$ ), while the affinity for substrate in mitochondrial fraction was around 10 times higher for poly  $P_3$  ( $K_{m,app} = 0.2 \mu\text{M}$ ) than for poly  $P_{15}$  ( $K_{m,app} = 2.8 \mu\text{M}$ ) and poly  $P_{65}$  ( $K_{m,app} = 3.6 \mu\text{M}$ ). PPX activity was stimulated by a factor of two by  $Mg^{2+}$  and  $Co^{2+}$  in the nuclear fraction and only by  $Mg^{2+}$  in the mitochondrial fraction. Heparin (20  $\mu\text{g}/\text{mL}$ ) inhibited nuclear and mitochondrial PPX activity in about 90 and 95% respectively. Together, these data are consistent with the existence of two different PPX isoforms operating in the nuclei and mitochondria of the hard tick *R. microplus* with distinct metal dependence, inhibitor and activator sensitivities. The data also shed new light on poly P biochemistry during arthropod embryogenesis, opening new routes for future comparative studies on the physiological roles of different poly P pools distributed over cell compartments.

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## 1. Introduction

Inorganic polyphosphates (poly P) are long chains of ten to many hundreds of phosphate residues linked by high-energy phosphoanhydride bonds. Poly P are ubiquitous, have been found in all cells examined (Kulaev and Kulakovskaya, 2000), and play varied roles, depending on the cell involved and on the circumstances (Kornberg et al., 1999).

The biologic functions of poly P have been studied most extensively in prokaryotes and unicellular eukaryotes, where it has already been described to regulate many biochemical processes such as: the energetic metabolism of  $P_i$ , cation sequestration and storage, formation of membrane channels,  $P_i$  transport, involvement in cell envelope formation and function, regulation of the gene and enzyme activities, involvement in bacterial pathogenesis, and activation of the Lon protease (Reusch and Sadoff, 1988; Kornberg et al., 1999; Rashid et al.,

2000; Kuroda et al., 2001; Kim et al., 2002; McInerney et al., 2006). On the other hand, poly P functions have not been extensively investigated in higher eukaryotes, although some functions have been described, such as activation of TOR kinase, involvement in blood coagulation, apoptosis, and the regulation of normal mitochondrial function in mammalian cells (Lorenz et al., 1997; Wang et al., 2003; Hernandez-Ruiz et al., 2006; Kawano, 2006; Smith et al., 2006; Abramov et al., 2007). Recently, we have reported that mitochondrial poly P is related to energy supply during *Rhipicephalus microplus* embryogenesis (Campos et al., 2007).

Poly P are hydrolyzed by exo and endopolyphosphatases. Exopolyphosphatases (PPX – polyphosphate-phosphohydrolases; EC 3.6.1.11) processively release the terminal phosphate groups from poly P formed by 3 phosphate residues and are considered as the central regulatory enzymes in poly P metabolism (Kulaev et al., 2000). However, the only gene encoding for a PPX (Wurst et al., 1995) that is known cannot account for all the different molecular masses, substrate specificities and requirements of divalent cations described for PPX activities (Kulaev et al., 2000). Based on the

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primary structure, PPXs are classified into two types, whose prototypes are PPXs from yeast (*Saccharomyces cerevisiae*) and *Escherichia coli*. Yeast-type PPX, reported in fungi and protozoa, belongs to the superfamily of DHH phosphoesterases (Aravind and Koonin, 1998), whereas *E. coli*-type PPX is present in Eubacteria and Archaea and belongs to a sugar kinase/actin/hsp-70 superfamily (Reizer et al., 1993).

The present study is focused on the tick *R. microplus*, which is a one-host tick that causes major losses to bovine herds, especially in tropical regions. In this scenario, major efforts have been made to develop immunoprophylactic tick control tools (Guerrero et al., 2006). Apart from this, the tick is the vector of parasites that cause hemoparasitic diseases, endemic in many cattle production areas (Sonenshine et al., 2006). *R. microplus* takes one host only throughout all three life stages, usually a bovine, with a long feeding period (approximately 21 days). The adult female, after completion of engorgement, drops off the host and initiates oviposition around 3 days later. Being an oviparous animal, embryogenesis occurs in the absence of exogenous nutrients, and maternal nutrients are packaged in oocytes and stored mostly as yolk granules. Hatching occurs around 21 days after oviposition, and the emerging larvae can survive several weeks before finding a host, using the remaining yolk as the only source of energy (Fagotto, 1990).

Vitellogenin is a hemolymphatic phospholipoproteoglycoprotein stored in growing oocytes. Once inside the oocytes, it is conventionally called vitellin and represents the main component of yolk platelets (Sappington and Raikhel, 1998). Most organisms contain vitellin covalently bound to  $P_i$ , which is the main source of  $P_i$  during embryogenesis (Sappington and Raikhel, 1998). More specifically, in *R. microplus* it has been demonstrated that vitellin is a phosphotyrosine-containing protein (Silveira et al., 2006).

The present study characterizes PPX activity in nuclear and mitochondrial fractions, in order to observe the importance of poly P metabolism in arthropod embryogenesis.

## 2. Materials and methods

### 2.1. Ticks

Ticks were obtained from a colony maintained at the Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Brazil. *R. microplus* (Acarina, Ixodidae) ticks from the Porto Alegre strain, free of parasites, were reared on calves obtained from a tick-free area. Engorged adult females were kept in Petri dishes at 28°C and 80% relative humidity upon completion of oviposition, which starts about 3 days after adult ticks drop off the calves.

### 2.2. Isolation of nuclei and mitochondria

The cell fractionation procedure used required large amounts of fresh eggs (at least 2 g) to obtain nuclear and functional active mitochondrial fractions. For the characterization of nuclear and mitochondrial fraction, eggs in the segmentation stage (9th day after oviposition) were used. Nuclei and mitochondria were isolated as previously described (Campos et al., 2007). Eggs were homogenized in 1.5 mL of a buffer containing 0.5 M sucrose, 100  $\mu$ M leupeptin, 100 nM pepstatin, 20 mM MgCl<sub>2</sub>, 10 mM EGTA, 1% bovine albumin (fat acid free) and 50 mM HEPES pH 7.2. The homogenate was centrifuged at 8000 g for 15 min. The supernatant was carefully removed and the pellet was resuspended in the homogenization buffer, and centrifuged at 500 g for 5 min. The supernatant was carefully removed again and centrifuged at 2000 g for 10 min to yield a nuclear pellet. Then, the supernatant was submitted to another centrifugation at 7000 g for 15 min, and the mitochondrial pellet that resulted therefrom was resuspended in a storage buffer containing 120 mM KCl, 1 mM EGTA, 0.2% bovine albumin, and 3 mM HEPES pH 7.2.

### 2.3. Extraction and determination of poly P content

Poly P was extracted from eggs homogenates, nuclei and mitochondria in three steps as previously described (Clark et al., 1986; Lorenz et al., 1997). Residual amounts of DNA and RNA were removed from the extracts obtained in steps 2 and 3 by treatment with RNase and DNase 1 (250  $\mu$ g/mL each) in the presence of 1 mM MgCl<sub>2</sub> for 1 h at room temperature. Protein was removed by one extraction with phenol/chloroform (1:1, v/v), followed by three successive extractions with chloroform. The poly P content in step 2 and step 3 extracts was estimated by determining the metachromatic effect of polyphosphate on the absorbance spectrum of toluidine blue at 530 nm and 630 nm (Clark et al., 1986; Lorenz et al., 1997). A calibration curve was obtained using poly P<sub>65</sub> (Sigma type 65) as a standard. The amount of poly P (plus  $P_i$ ) in step 1 extracts and of residual poly P not extracted by steps 2 and 3 was determined after hydrolysis at 100°C (Lorenz et al., 1997). The resulting  $P_i$  was measured spectrophotometrically as described by Fiske and Subbarow (1925).

### 2.4. PPX activity, $P_i$ and protein assay

The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), and 5 mM MgCl<sub>2</sub>, using poly P<sub>3</sub>, poly P<sub>15</sub> or poly P<sub>65</sub> as substrate. Reactions were carried out at 30°C for various time periods. The  $P_i$  formed during the reaction was spectrophotometrically determined as previously described by Fiske and Subbarow (1925), by adding a solution of 0.5% ammonium molybdate, 0.35 M sulfuric acid, 0.5% SDS, and 10% ascorbic acid. Measurements of absorbance at 750 nm were performed after 15 min. The enzyme amount liberating 1  $\mu$ mol of  $P_i$ /min was defined as one unit of enzyme activity (U). Protein concentration was measured by Bradford (1976), using bovine serum albumin as a standard.

### 2.5. Extraction of total RNA

Total RNA was extracted from 50 mg of egg homogenates with Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions and precipitated with 0.5 mL isopropanol for 2 h at -20°C. Subsequently, the extracted RNA was washed with 1 mL 75% ethanol, vacuum-dried for 10 min and resuspended in water DEPC. Absorbance was measured at 260 and 280 nm, assuming that one absorbance unity at  $A_{260}$  corresponds to 40  $\mu$ g/mL RNA (Sambrook et al., 1989).

### 2.6. Electrophoresis of poly P

The poly P preparation obtained was electrophoresed on 7 M urea/20% polyacrylamide gels (30 × 15 × 0.3 cm; bisacrylamide:acrylamide ratio, 1:20) in TBE (90 mM Tris, 90 mM borate, 2.7 mM EDTA, pH 8.3). Gels were stained with toluidine blue as previously described (Clark et al., 1986; Lorenz et al., 1994).

### 2.7. F-ATPase activity

The F-ATPase (EC 3.6.14); azide sensitive activity of the mitochondrial fraction was spectrophotometrically measured as described by Li and Neufeld (2001). The activity was assayed at 30°C and the reaction mixture contained 50 mM Tris-HCl (pH 8.0), 2 mM MgCl<sub>2</sub>, 100 mM KCl, 2 mM ATP. As inhibitor, 5 mM azide was used. The enzyme amount liberating 1  $\mu$ mol of  $P_i$ /min was taken as a unit of enzyme activity (U). The  $P_i$  formed was determined as described by Fiske and Subbarow (1925).

### 2.8. Glucose-6-phosphate dehydrogenase activity

G6PDH (EC 1.1.1.49) activity was determined according to Worthington (1988). G6PDH activity was obtained by determining

the rate of NADPH formation at 30°C, which absorbs ultraviolet light at 340nm, using a Beckman DU640 spectrophotometer.

### 2.9. Respiration measurements

The rate of O<sub>2</sub> consumption by mitochondria was estimated by a Clark oxygen electrode (YSI, mod. 5775, Yellow Springs, OH). The calibration process was conducted using the initial O<sub>2</sub> concentration of the medium as 100% – saturated buffer measured at 28°C. Measurements were carried out in 1.5mL reaction buffer containing 120mM KCl, 1mM EGTA, 0.2% bovine albumin, 3mM HEPES (pH 7.2), 2.5mM phosphate and 1mM KCN or 2.5µg/mL oligomycin was used as inhibitor. Mitochondrial protein (0.5mg/mL) was used, and after a 1-min equilibration period, mitochondrial respiration was started by the addition of pyruvate to a final concentration of 5mM (Campos et al., 2007). Rates of oxygen consumption are expressed as nmol O<sub>2</sub>/(mg mitochondrial protein min). The respiratory control ratio (RCR) was defined as the rate of ADP (0.5mM)-stimulated oxygen consumption (State 3) divided by the rate of respiration determined in the presence of oligomycin (2.5µg/mL) (State 4<sub>o</sub>), an inhibitor of ATP synthase. While the State 4<sub>o</sub> respiration measured in the presence of oligomycin is not equivalent to the classical State 4 rate obtained after a small bolus of ADP has almost completely been converted to ATP, the use of oligomycin eliminates the contribution of ATP cycling via hydrolysis by contaminating ATPases and resynthesis by the mitochondrial ATP synthase to State 4 respiration. The oligomycin-induced State 4<sub>o</sub> rate of respiration is therefore a more specific indicator of mitochondrial proton cycling limited by passive proton leakiness of the inner membrane (Kristian et al., 2006).

### 2.10. Statistical analysis

All data values were expressed as mean ± S.D. ANOVA was used to determine significant differences between groups when data were normally distributed. The t-tests were used when comparing data between two groups. Significance was set at  $p < 0.05$  (Swinscow, 1996).

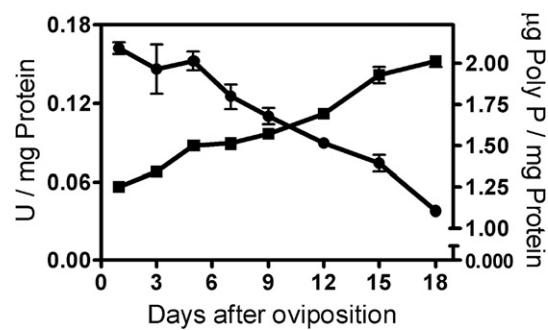
## 3. Results

### 3.1. Nuclei and mitochondria isolation

The purity and intactness of the nuclear fraction was rather satisfactory, as determined by examination in the phase-contrast (data not shown). The nuclear purity was also biochemically characterized by the absence of marker enzymes of the other compartment. In the isolated nuclear fraction, no activity of either F-ATPase azide sensitive (a mitochondrial marker), or glucose-6-phosphate dehydrogenase (a cytosol marker) could be detected (Table 1). In the mitochondrial fraction, when oxidizing pyruvate, O<sub>2</sub> consumption was 29.7nmol O<sub>2</sub>/min mg protein, and RCR was 6.8. This process was KCN- and oligomycin-sensitive and ATP hydrolysis azide-sensitivity was higher than 80% (Table 1).

**Table 1**  
Characterization of the cell fractionation of *Rhipicephalus microplus* embryos on the 9th day of embryogenesis

|                           | ATPase activity<br>(U/mg protein) | G6PDH<br>(U/mg protein) | O <sub>2</sub> consumption<br>(nmol min <sup>-1</sup> mg<br>protein <sup>-1</sup> ) | RCR | % F-ATPase<br>azide<br>sensitive |
|---------------------------|-----------------------------------|-------------------------|-------------------------------------------------------------------------------------|-----|----------------------------------|
| Homogenate                | 0.7                               | 2.9                     | –                                                                                   | –   | 49.5%                            |
| Nuclear<br>fraction       | –                                 | –                       | –                                                                                   | –   | –                                |
| Mitochondrial<br>fraction | 1.5                               | –                       | 29.7                                                                                | 6.8 | 83.4%                            |



**Fig. 1.** PPX activity and poly P content of nuclear fraction during *R. microplus* embryogenesis. Activity is expressed as U/mg of total protein in the nuclear fraction preparations. (■) PPX activity was measured with 2.5 mM poly P<sub>15</sub>, 0.1 mM Co<sup>2+</sup> and 5 mM MgCl<sub>2</sub>. (●) Poly P levels during embryogenesis in the nuclear fraction preparations. The results represent mean±S.D. of three independent experiments, in triplicates.

### 3.2. Characterization of nuclear and mitochondrial poly P content in *R. microplus* embryogenesis

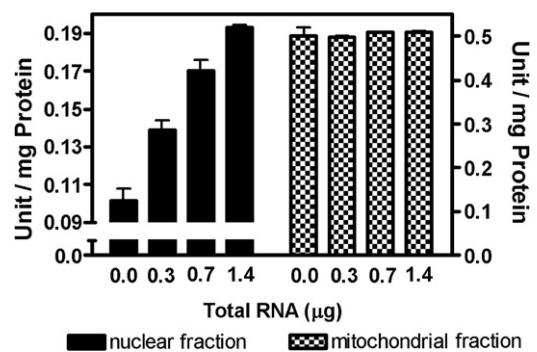
The poly P content and PPX activity were measured using the nuclear fraction isolated from the *R. microplus* eggs during embryogenesis. Nuclear PPX activity increased throughout embryogenesis. Poly P levels decreased after embryo cellularization (i.e. 5th day of development), reflecting PPX activity (Fig. 1). The mobilization in mitochondrial poly P content is different, where a higher poly P utilization occurred during blastoderm formation and segmentation of the embryo, between the 5th and 7th day of development, as we previously reported (Campos et al., 2007).

The influence of total RNA in nuclear and mitochondrial PPX activity was investigated in concentrations that ranged from 0.3 to 1.4µg. Nuclear PPX activity was stimulated by a factor of two, while mitochondrial PPX activity did not change (Fig. 2).

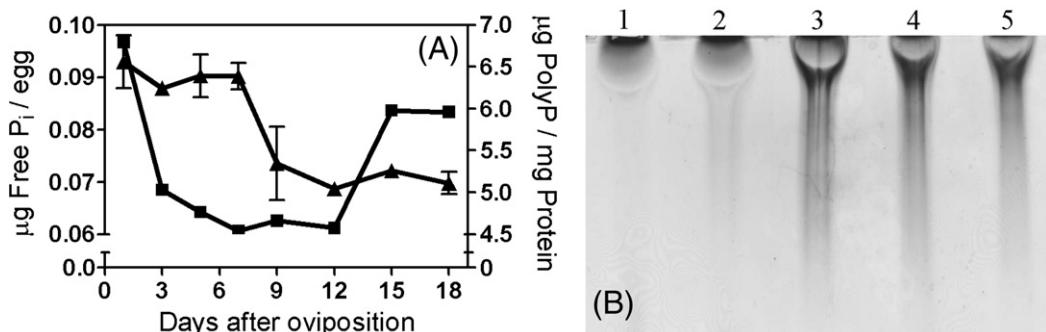
### 3.3. Characterization of total poly P content during *R. microplus* embryogenesis

Total poly P levels were quantified throughout *R. microplus* embryogenesis. Total poly P levels were higher during embryo cellularization and segmentation, from the 5th to 7th day of development, and declined after that until reaching a plateau. Free P<sub>i</sub> content rapidly decreased during syncytial blastoderm formation, on the 3rd day of development, continuing low until the 12th of embryogenesis, when it's rapidly increased thereafter (Fig. 3A).

Total poly P extracted on different days of embryogenesis was electrophoresed by 20% urea-PAGE to compare their size throughout



**Fig. 2.** Influence of total RNA in PPX activity of nuclear and mitochondrial fractions. Nuclei and mitochondria of eggs on the 9th day of embryogenesis were isolated, and PPX activity was determined using poly P<sub>15</sub> as substrate in the presence of 0.3 to 1.4 µg of total RNA. The results represent mean±S.D. of three independent experiments, in triplicates.



**Fig. 3.** Characterization of total poly P content in *R. microplus* embryogenesis. In (A): total poly P (▲) was extracted and quantified and free  $P_i$  (■) was quantified in eggs homogenate in different days after oviposition. The results represent mean  $\pm$  S.D. of three independent experiments, in triplicates. In (B): Electrophoresis was performed on a 7 M urea/20% polyacrylamide gel. The gel was stained with toluidine blue. Equal amounts of extracts were applied per lane. Lanes 1, 2, 3, 4 and 5 contain total poly P extracted from eggs on the 1st, 5th, 9th, 15th, and 18th day after oviposition, respectively. This experiment was repeated at least three times with different preparations and this figure shows a representative experiment.

embryogenesis of *R. microplus*. The chain length of total poly P content decreased after embryo cellularization, on the 5th day of development (Fig. 3B).

#### 3.4. Characterization of nuclear and mitochondrial PPX

The apparent  $K_m$  was measured for nuclear and mitochondrial PPX using poly  $P_3$ , poly  $P_{15}$  and poly  $P_{65}$  as substrates in eggs in the segmentation stage (9th day after oviposition). The mitochondrial affinity PPX for poly  $P_3$  was as great as 10 times that for poly  $P_{15}$  or for poly  $P_{65}$ ; on the other hand, nuclear PPX had almost the same affinity for all substrates (Table 2). The dependence of hydrolysis rates of poly P in the presence of 2.5 mM  $Mg^{2+}$  and 0.1 mM  $Co^{2+}$  was in agreement with Michaelis kinetics. The nuclear fraction was stimulated by both cations, while the mitochondrial fraction was stimulated only by  $Mg^{2+}$ . Yet, no stimulation was detected using  $Co^{2+}$  (Fig. 4). Heparin, a good inhibitor for other PPX (Kornberg et al., 1999), was also effective against these PPX, as 20  $\mu$ g/mL heparin inhibited nuclear and mitochondrial PPX activity by about 90 and 95%, respectively (Fig. 5).

#### 4. Discussion

Although the first evidence for the presence of poly P in mammalian cells was obtained a long time ago (Gabel and Thomas, 1971), since then relatively few works have addressed their physiological roles in animal cells (Kornberg et al., 1999; Wang et al., 2003; Hernandez-Ruiz et al., 2006; Kawano, 2006; Smith et al., 2006; Abramov et al., 2007; Campos et al., 2007). In this study, we have compared poly P metabolism between nuclear and mitochondrial fractions. The results indicate different mobilizations in these fractions during the whole embryogenesis stage, suggesting distinct and important functions of poly P in tick embryo development.

Early *R. microplus* embryonic stages are similar to those of *D. melanogaster* and of mosquitoes (Bate and Arias, 1991; Monnerat et al., 2002). Tick embryogenesis is characterized by the formation of a non-cellular syncytium up to day 4 (Campos et al., 2006). After this, the embryo becomes a multicellular organism and starts organogenesis (Campos et al., 2006).

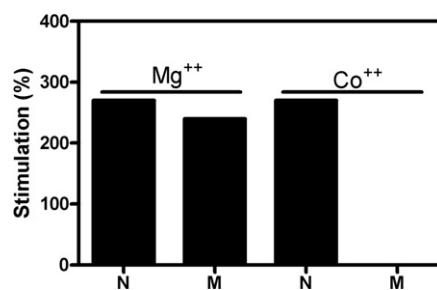
**Table 2**

Apparent  $K_m$  values of the PPX in nuclear and mitochondrial fractions of *R. microplus* embryos on 9th day of embryogenesis

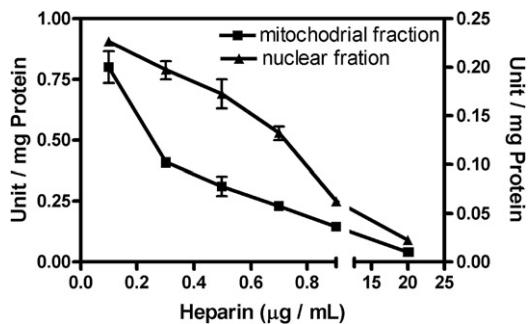
| Substrate     | Nuclear fraction | Mitochondrial fraction |
|---------------|------------------|------------------------|
|               | $K_m$ ( $\mu$ M) |                        |
| Poly $P_3$    | 0.7              | 0.2                    |
| Poly $P_{15}$ | 1.1              | 2.8                    |
| Poly $P_{65}$ | 0.9              | 3.6                    |

The poly P in different cell compartments was best studied in *S. cerevisiae*, and has been proved to depend on culture age and cultivation conditions (Kulaev, 1975; Kulaev and Kulakovskaya, 2000; Lichko et al., 2003). The participation of nuclear poly P in the multiple steps that occur along the pathway from gene to protein is one of the most important functions of these compounds in eukaryotic and prokaryotic microorganisms, and it is well documented in controlling gene expression, as in the adaptation to stationary phase or other developmental transitions, for example (Brown and Kornberg, 2004). Additionally, it has been demonstrated that poly P is involved in several aspects of mRNA transcription, and that purified poly P physically interacts with purified RNA polymerase (Kusano and Ishihama, 1997). Poly P has also been implicated in mRNA stability (Blum et al., 1997). Similarly, it has been shown that an increase in protein production is caused by poly P (Itoh et al., 2006), and that an enhancement in polysome stability *in vivo* prevents miscoding events during protein synthesis (McInerney et al., 2006). In nuclei of rat liver, poly P is related to the fraction of non-histone proteins (Offenbacher and Kline, 1984). Poly P may interact with DNA-histone binding in chromatin, and this binding has been shown to inhibit the activity of some nuclear enzymes, including topoisomerases (Schroder et al., 1999). These data support the hypothesis that poly P are involved in the regulation of chromatin functions in higher eukaryotes.

The results obtained demonstrate that poly P is present in the nuclei of *R. microplus* embryos, and that nuclear poly P content reflects the nuclear PPX activity during embryogenesis (Fig. 1). The data suggest that the mobilization of the nuclear fraction is completely different from that of the mitochondrial fraction, as shown in a previous work (Campos et al., 2007). During embryo cellularization, the nuclear fraction showed higher polyphosphate levels (9 to 10 times) in comparison to the mitochondrial fraction. Similarly, the



**Fig. 4.** Effect of divalent cations on PPX activity in nuclear and mitochondrial fractions of *R. microplus* embryos. Nuclei (N) and mitochondria (M) of eggs on the 9th day of embryogenesis were isolated and the PPX activity was determined using poly  $P_{15}$  as substrate in the presence of 2.5 mM  $Mg^{2+}$  and 0.1 mM  $Co^{2+}$ . The results represent mean  $\pm$  S.D. of three independent experiments, in triplicates.



**Fig. 5.** Effect of heparin on PPX activity in nuclear and mitochondrial fractions of *R. microplus*. Nuclei (N) and mitochondria (M) of eggs on the 9th day of embryogenesis were isolated and the PPX activity was determined using poly P<sub>15</sub> as substrate and heparin in a concentration range of 0.1–20 μg/mL. The results represent mean±S.D. of three independent experiments, in triplicates.

same has been described for nuclear and mitochondrial fractions in rat liver (Kumble and Kornberg, 1995). In the present study, nuclear poly P decreased while total RNA increased, after the formation of a cellular syncitium (6th day of development), as also demonstrated in previous research (Campos et al., 2006). Thus, nuclear poly P levels seemed to correlate with total RNA content during *R. microplus* embryogenesis. Yet, contrasting with the mitochondrial PPX activity, only the nuclear activity is stimulated by total RNA (Fig. 2).

The function of poly P as P<sub>i</sub> reserve is well known in prokaryotes and also in eukaryote microorganisms (Kulaev, 1975; Kulaev and Vagabov, 1983; Kornberg, 1995). The cells of higher eukaryotes carry poly P, but in smaller amounts than those found in microorganisms. So, beyond the source of P<sub>i</sub>, these biopolymers probably participated in regulatory processes (Kornberg et al., 1999). It is interesting to note that in *R. microplus*, the decline in total poly P content after the 7th day of embryogenesis does not reflect the increase in free P<sub>i</sub>, since this occurs only after the 12th day, suggesting that poly P also plays other roles, apart from the P<sub>i</sub> reserve for embryo development (Fig. 3A). In this case, an alternative source of P<sub>i</sub> could be the dephosphorylation of vitellin, a major yolk protein that is gradually dephosphorylated during embryogenesis (Silveira et al., 2006). This source can be used mainly until the segmentation of embryo, 7th day of development, because in this period there is no total poly P mobilization. The decrease in chain length after embryo segmentation (9th day of embryogenesis) is confirmed by electrophoresis of total poly P content, which compared poly P chain lengths during embryogenesis (Fig. 3B).

Additionally, here we compare the kinetic parameters of nuclear and mitochondrial fraction. PPXs have been found in prokaryotes and eukaryotes and although in bacteria these enzymes hydrolyze mostly high molecular weight poly P (Kumble and Kornberg, 1996), at least some of the enzymes from *S. cerevisiae* and *Leishmania major* are more active in hydrolyzing short chain poly P, such as poly P<sub>3</sub> (Kumble and Kornberg, 1996; Rodrigues et al., 2002). Our results show that nuclear PPX activities with poly P<sub>3</sub>, poly P<sub>15</sub> and poly P<sub>65</sub> were nearly the same; however, affinity of mitochondrial PPX for poly P<sub>3</sub> was around 10 times higher than that for poly P<sub>15</sub> and poly P<sub>65</sub> (Table 2). To our knowledge, the *R. microplus* nuclear PPX is the only enzyme that hydrolyzes short and long polyphosphate with the same affinity. PPX from *E. coli* requires divalent cations and K<sup>+</sup> for maximal activity, while PPX from yeast requires only divalent cations (Lichko et al., 2003). The nuclear PPX activity is stimulated by Mg<sup>2+</sup> and Co<sup>2+</sup>, while the mitochondrial PPX activity is stimulated only by Mg<sup>2+</sup> (Fig. 4), and both are insensitive to K<sup>+</sup> (data not shown). These differences confirm and reflect the different poly P functions of these fractions for embryo development.

Only few compounds that inhibit PPX have been identified (Kornberg et al., 1999). Treatment with molybdate (a common phosphohydrolase inhibitor), and fluoride (a pyrophosphatase inhibitor) showed that PPX

present in nuclear and mitochondrial fractions are insensitive to these compounds (data not shown). However, heparin, a good inhibitor of other well characterized PPX (Lichko et al., 2003), was effective in both fractions, and the kinetics of inhibition was different between the fractions, the mitochondrial fraction being more sensitive than the nuclear fraction (Fig. 5). Together, these results confirmed the presence of two different PPXs specific for each one of the fractions studied, since both showed differential sensitivity for inhibitors and different kinetic characteristics for each cell compartment.

The ubiquity of poly P and variations in its chain length, location, and metabolism indicate the relevant functions this polymer plays, also in animal systems. The results suggest that inorganic polyphosphate is involved in the mitochondrial and nuclear metabolism in *R. microplus* embryogenesis. In nuclei, PPX activity was activated by RNA and thus should be involved in the RNA metabolism. In contrast, the mitochondria poly P was insensitive to RNA, which seems to be related to energy supply (Campos et al., 2007). The present work clearly demonstrates that these two PPX activities represent at least two different PPX enzymes with distinct metal dependence, inhibitor and activators sensitivities. In this sense, a more comprehensive understanding of poly P biochemistry during arthropod embryo development may support to unravel additional targets that could be effective in the control of this ectoparasite, and shed new light on poly P metabolism.

## Acknowledgments

We are grateful to Cristóvão Barros Pinheiro for his excellent technical assistance. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ, Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, Programa de Núcleos de Excelência – PRONEX and Programa Nacional de Cooperação Acadêmica – PROCAD-CAPES.

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## Short communication

# A mitochondrial exopolyphosphatase activity modulated by phosphate demand in *Rhipicephalus (Boophilus) microplus* embryo

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Received 23 April 2007; received in revised form 5 June 2007; accepted 12 June 2007

## Abstract

This study describes Exopolyphosphatases (PPX) activity in mitochondria of *Rhipicephalus microplus* embryos. Mitochondria were isolated by differential centrifugation and PPX activity was analyzed through the hydrolysis of the substrate Polyphosphate (Poly P<sub>15</sub>). We investigated the influence of NADH, NAD<sup>+</sup>, P<sub>i</sub> and ADP in a concentration range of 0.1–2.0 mM. Poly P hydrolysis was stimulated about two-fold by NADH and strongly inhibited by P<sub>i</sub>. The PPX activity also increased in the presence of the respiratory substrates pyruvic and succinic acids, and this stimulatory effect disappeared upon addition of KCN. Mitochondrial respiration was activated by ADP using poly P as the only source of P<sub>i</sub>. Endogenous poly P content changed following PPX activity during embryogenesis from the first up to 18th day of development. The data describe exopoly P as being modulated by P<sub>i</sub> demand and related to energy supply during embryogenesis of hard ticks.

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**Keywords:** Polyphosphate; Bioenergetics; Phosphate demand; *Rhipicephalus boophilus microplus*; Arthropoda

## 1. Introduction

Inorganic linear polyphosphates (poly P) are energy-rich polymers of up to several hundreds of orthophosphate (P<sub>i</sub>) residues, and are found in bacteria, fungi, plants and animals (Kornberg et al., 1999). Poly P is best characterized in bacteria and yeast cells, where it has already been described to regulate many biochemical processes such as: the energetic metabolism of P<sub>i</sub>, cation sequestration and storage, formation of membrane channels, P<sub>i</sub> transport, involvement in cell envelope formation and function, regulation of the gene and enzyme activities, involvement in bacterial pathogenesis and activation of the Lon protease (Kornberg et al., 1999; Rashid et al., 2000; Kuroda et al., 2001; Kim et al., 2002; McInerney et al., 2006). Poly P functions have not been extensively

investigated in higher eukaryotes, although some functions were described such as activation of TOR kinase, involvement in blood coagulation and apoptosis in mammalian cells (Lorenz et al., 1997; Wang et al., 2003; Smith et al., 2006; Hernandez-Ruiz et al., 2006; Kawano, 2006).

Poly P metabolism in eukaryotic cells shows specific peculiarities for different cellular compartments. Physiological studies include the development of mutants deficient in enzymes, mainly from *Saccharomyces cerevisiae* and *Escherichia coli* (Akiyama et al., 1993; Ogawa et al., 2000; Freimoser et al., 2006). Exopolyphosphatases (PPX; polyphosphate-phosphohydrolases; EC 3.6.1.11) split P<sub>i</sub> off the end of a poly P chain, while endopolyphosphatases (PPN; polyphosphate-polyphosphohydrolase; EC 3.6.1.10) cleave the phosphoanhydride bounds inside the poly P chain. These are the main enzymes responsible for poly P hydrolysis (Kornberg et al., 1999). Mitochondria of *S. cerevisiae* possess their own pool of poly P (Pestov et al., 2005), which has been described as a potential P<sub>i</sub> source

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that can be used for oxidative phosphorylation (Beauvoit et al., 1989). At least two PPX have been found in *S. cerevisiae* mitochondria, and a vacuolar localization for PPN has been proposed (Huh et al., 2003). The *PPN1* gene has been found to have a substantial effect on the PPX spectrum in the cytosol and yeast mutants with inactivation of PPN has a defect in mitochondrial respiration activity (Pestov et al., 2005).

The present study focused on the tick *Rhipicephalus (Boophilus) microplus*, which is an important cattle ectoparasite in South and Central Americas, Asia, Africa and Oceania (Leal et al., 2006). This ixodidae tick is recognized worldwide as vector of several parasitic protozoa (Johnston et al., 1986). *R. microplus* is a one-host tick, with larval, nymphal and adult stages that feed in sequence on the same host, usually a bovine, for approximately 21 days. The adult female, after completion of engorgement, drops off the host and initiates oviposition 3 days later. As an oviparous animal, it faces embryogenesis in the absence of exogenous nutrients. Thus, maternal nutrients are packaged into the oocytes and stored mostly as yolk granules. Hatching occurs around 21 days after egg laying and the resulting larvae can survive several weeks before finding a host, using the remained yolk as the only source of energy (Fagotto, 1990). Vitellogenin is a hemolymphatic phospholipoplycoprotein stored in growing oocytes. Once inside the oocytes, it is conventionally called vitelin (VT) and represents the main component of yolk platelets (Sappington and Raikhel, 1998). Most organisms contain VT covalently bound to  $P_i$ , which is the main source of  $P_i$  during embryogenesis (Sappington and Raikhel, 1998), and in *R. microplus* it has been demonstrated that VT is a phosphotyrosine-containing protein (Silveira et al., 2006).

The present study investigates the regulation of mitochondrial PPX activity during embryogenesis of *R. microplus*. Data are discussed in terms of a role for poly P in the energy metabolism during tick embryogenesis regulated by  $P_i$  and energy demand.

## 2. Materials and methods

Ticks were obtained from a colony maintained at the Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Brazil. *R. microplus* ticks from the Porto Alegre strain, free of Babesia spp., were reared on calves obtained from a tick-free area. Engorged adult females were kept in Petri dishes at 28 °C and 80% relative humidity upon completion of oviposition, which starts about 3 days after dropping off the calves.

Poly P was extracted from egg homogenates or mitochondrial fraction in three steps as previously described (Lorenz et al., 1997). The content of poly P in steps 2 and 3 of poly P extraction was estimated by measuring the metachromatic effect produced by poly P on the absorption spectrum of toluidine blue, as described in (Lorenz et al., 1997), and a calibration curve was obtained using poly  $P_{65}$  as standard. The amounts of poly P (plus  $P_i$ )

in step 1 and of residual poly P not extracted in steps 2 and 3 were determined after acid hydrolysis at 100 °C (Lorenz et al., 1997). The resulting  $P_i$  was spectrophotometrically measured as previously described (Malpartida and Serrano, 1981).

PPX activities were determined by the rate of  $P_i$  formation at 30 °C for different periods of time, using poly  $P_{15}$  as substrate (Lorenz et al., 1997). The F-ATPase azide sensible activity of the mitochondrial fraction was spectrophotometrically measured as described by (Li and Neufeld, 2001). The resulting  $P_i$  was spectrophotometrically determined with ascorbic acid and a solution containing ammonium molybdate and SDS (Malpartida and Serrano, 1981). The amount of enzyme that releases 1 μmol of  $P_i$  per 1 min was defined as one unit of enzyme activity (U). Protein concentration was measured according to Bradford (1976), using bovine serum albumin as a standard.

For isolation of mitochondria, eggs were homogenized in a buffer containing 0.5 M sucrose, 100 μM leupeptin, 100 nM pepstatin, 1 mM PMSF, 20 mM MgCl<sub>2</sub>, 10 mM EGTA, 1% bovine albumin (fat acid free) and 50 mM HEPES pH 7.2. The homogenate was centrifuged at 8000g for 15 min. The supernatant was carefully removed and the pellet was re-suspended in the homogenization buffer and centrifuged at 500g for 5 min. The supernatant was carefully removed again and centrifuged at 2000g for 10 min to yield a nuclear pellet. Then, the supernatant was submitted to another centrifugation at 7000g for 15 min and the mitochondrial pellet that resulted therefrom was re-suspended in a storage buffer containing 120 mM KCl, 1 mM EGTA, 0.2% bovine albumin, and 3 mM HEPES pH 7.2.

The rate of O<sub>2</sub> uptake by mitochondria was estimated by a Clark oxygen electrode (YSI, mod. 5775, Yellow Springs, OH). The calibration process was conducted using the initial O<sub>2</sub> concentration of the medium as 100%—saturated buffer measured at 28 °C. Measurements were carried out in 1.5 mL reaction buffer containing 120 mM KCl, 1 mM EGTA, 0.2% bovine albumin, and 3 mM HEPES (pH 7.2) in the absence of any  $P_i$  source containing 0.5 mg/mL of mitochondrial protein. After a 1-min equilibration period, mitochondrial respiration was started by addition of pyruvate to a final concentration of 5 mM. Each experiment was repeated at least three times with different mitochondrial preparations. Fig. 2B shows representative experiments, and other additions are indicated in the figure legend (Pestov et al., 2004).

The PPX activity during mitochondrial respiration was measured using a reaction mixture that consisted of 50 mM Tris-HCl buffer (pH 7.2), 120 mM KCl, 1 mM EGTA, 5 mM MgCl<sub>2</sub> and 0.2 mM ADP in the absence of any  $P_i$  source. Poly  $P_{15}$  (5 mM) was used as substrate for PPX activity, and 5 mM pyruvate and 3 mM succinate were used as oxidative substrates. Cyanide (1 mM KCN) was used to inhibit the cytochrome oxidase. The reaction was carried out at 28 °C for 15 min (Pestov et al., 2004). The  $P_i$  formed

during the reaction was spectrophotometrically determined (Malpartida and Serrano, 1981).

### 3. Results

This study describes a mitochondrial preparation from tick eggs. The cell fractionation procedure used required large amounts of fresh eggs (at least 2 g) to obtain functional active mitochondrial fractions. Table 1 shows an enrichment of 165% of the mitochondrial fraction, and this fraction exhibited an ATP hydrolyses azide sensitivity higher than 80%, which is related to the F-ATPase activity, a mitochondria marker.

Mitochondria of the eggs in the segmentation stage (9th day after oviposition) were isolated, and the PPX activity was measured in order to evaluate the regulation of the activity of this enzyme. The influence of NADH,  $\text{NAD}^+$ ,  $\text{P}_i$  and ADP was investigated in concentrations that ranged from 0.1 to 2.0 mM. In the absence of  $\text{P}_i$ , PPX activity was stimulated by a factor of two by NADH, while  $\text{NAD}^+$

barely changed this activity (Fig. 1A). The activity was completely inhibited with 2 mM  $\text{P}_i$  (Fig. 1B), and slightly stimulated by ADP (Fig. 1C).

The mitochondrial PPX activity was measured during cellular respiration using pyruvate or succinate as substrates, and the oxygen consumption was monitored using poly P as the only source of  $\text{P}_i$ . PPX activity increased around 17% and 25% using pyruvate and succinate, respectively. When mitochondrial respiration was inhibited by KCN, the stimulatory effects of both respiratory substrates disappeared (Fig. 2A). The addition of 5 mM Poly  $\text{P}_{15}$  stimulated oxygen consumption and when a new state four was established, 5 mM  $\text{P}_i$  was added, thus stimulating again oxygen consumption (Fig. 2B).

Mitochondrial poly P was quantified and PPX was measured throughout embryogenesis of *R. microplus*. PPX activity was higher during embryo cellularization and segmentation, from the 5th to 7th day of development, and at the same time poly P levels declined (Fig. 3). Total poly P was also quantified throughout embryogenesis, with

Table 1  
F-ATPase activities in the cell fractionation of *Rhipicephalus (Boophilus) microplus* embryos on 9th day of embryogenesis

|                        | Total activity<br>(U/mg protein) | Total protein<br>(mg/mL) | Specific activity<br>(U/mg protein) | Enrichment (%) | F-ATPase azide<br>sensitive (%) |
|------------------------|----------------------------------|--------------------------|-------------------------------------|----------------|---------------------------------|
| Homogenate             | 0.7613                           | 3.98                     | 0.3769                              | —              | 49.50                           |
| Nuclear fraction       | 0.0000                           | 2.81                     | 0.0000                              | —              | 0                               |
| Mitochondrial fraction | 1.5041                           | 1.30                     | 1.2552                              | 165            | 83.45                           |

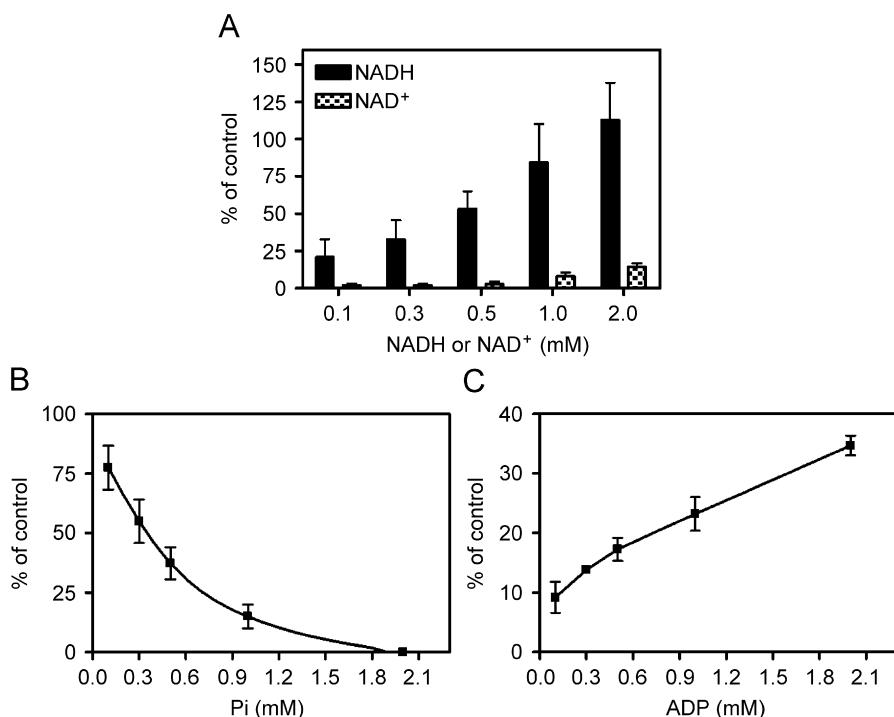


Fig. 1. Mitochondrial PPX activity in *R. microplus* embryos. Mitochondria of eggs on the 9th day of embryogenesis were isolated, and PPX activity was determined using poly  $\text{P}_{15}$  in the presence of 0.1–2 mM (A) NADH and  $\text{NAD}^+$ , (B) ADP and (C)  $\text{P}_i$ . The results represent mean  $\pm$  SD of three independent experiments, in triplicates.

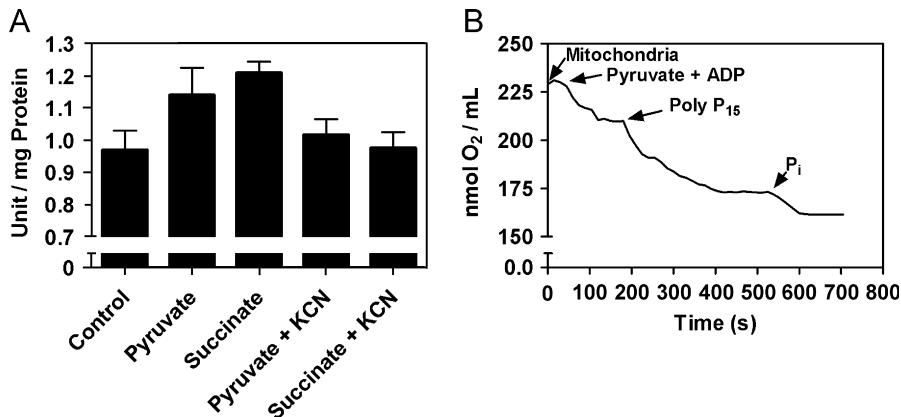


Fig. 2. Poly P as source of  $P_i$  for ATP synthesis. In (A), PPX activity was measured in mitochondria of the eggs on the 9th day of development during mitochondrial respiration, using pyruvate or succinate as oxidative substrates and KCN as inhibitor of the respiratory chain. In (B), oxygen consumption was monitored using a reaction buffer in the absence of a  $P_i$  source. The addition of 0.2 mM ADP, 5 mM pyruvate, 5 mM poly P and 5 mM  $P_i$  is represented in the figure. The results represent mean  $\pm$  SD of three independent experiments, in triplicates.

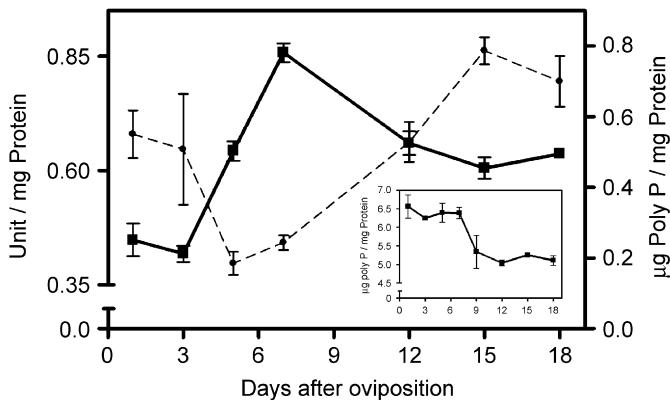


Fig. 3. Polyphosphate metabolism during *R. microplus* embryogenesis. Activity is expressed as units per milligram of total protein in the mitochondrial fraction preparations. (■) PPX activity was measured with 2.5 mM poly  $P_{15}$  and 5 mM  $MgCl_2$ . (●) Poly P levels during embryogenesis in the mitochondrial fraction preparations. Inset represents the levels of total poly P content during embryogenesis. The results represent mean  $\pm$  SD of three independent experiments, in triplicates.

poly P content being higher during embryo cellularization and segmentation, from the 5th to 7th day of development and decreasing after that (Fig. 3, inset).

#### 4. Discussion

This work reports a PPX activity that was stimulated 100% by NADH, 35% by ADP and completely inhibited by 2 mM  $P_i$  (Figs. 1A–C). These data suggest that mitochondrial PPX activity is regulated by  $P_i$  and energy demand. PPX activity increased accordingly during mitochondrial respiration when either pyruvate or succinate was added, and its stimulatory effect disappeared upon the addition of KCN, an inhibitor of the oxidative phosphorylation (Fig. 2A). Furthermore, it was possible to measure ADP-dependent mitochondrial oxygen consumption in the presence of poly P and in the absence of any other source

of  $P_i$ , which supports the hypothesis that poly P has been used as a  $P_i$  donor for ATP synthesis (Fig. 2B).

Uncouplers inhibited poly P accumulation in *S. cerevisiae* mitochondria under phosphate limitation and fast hydrolysis of poly P was observed after sonication of isolated mitochondria (Pestov et al., 2004, 2005). This suggests that the accumulation of poly P depends on the mitochondrial membrane proton-motive force (Pestov et al., 2005).

In order to gain insights into the possible physiological role of poly P metabolism, poly P levels and PPX activities were analyzed throughout embryogenesis. Early *R. microplus* embryonic stages are typical of the *D. melanogaster* model system, and have also been observed in mosquitoes (Bate and Arias, 1991; Monnerat et al., 2002). Tick embryogenesis is characterized by the formation of a non-cellular syncitium up to day 4 (Campos et al., 2006). After this, the embryo becomes a multicellular organism and starts organogenesis (Campos et al., 2006). A higher PPX activity associated with a higher poly P utilization in mitochondrial fraction occurred during the blastoderm formation and segmentation of the embryo, between the 5th and 7th day of development (Fig. 3) and a higher total poly P utilization occurred after blastoderm formation and segmentation of the embryo, after the 7th day of development (Fig. 3, inset). Thus, PPX activity and mitochondrial poly P levels seemed to correlate with the energy demand of the embryo during these developmental stages, in which the embryo utilizes a large part of its reserve lipids and sugars (Campos et al., 2006). After the 7th day, the mitochondrial poly P content increases, while PPX activity decreased upon reaching a stable plateau between the 12th and 18th day of development. These data suggest that in the late stages, the balance between synthesis and hydrolysis of mitochondrial poly P changes. In this case, an alternative source of  $P_i$  at these stages may be the poly P content of other cell compartments, because the mitochondrial poly P levels increase while total poly

P levels decrease (Fig. 3). Another possibility is the dephosphorylation of vitelin, a major yolk protein that is gradually dephosphorylated during embryogenesis (Silveira et al., 2006).

In conclusion, we provide the first compelling biochemical evidences for the presence of a mitochondrial poly P metabolism in an arthropod and describe a role for poly P in energy supply during embryogenesis of the hard tick *R. microplus*.

## Acknowledgments

We are grateful to Cristóvão Barros Pinheiro for his excellent technical assistance. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro—FAPERJ, Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq, Programa de Núcleos de Excelência—PRONEX and Programa Nacional de Cooperação Acadêmica—PROCAD-CAPES.

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# Relationship between glutathione *S*-transferase, catalase, oxygen consumption, lipid peroxidation and oxidative stress in eggs and larvae of *Boophilus microplus* (Acarina: Ixodidae)<sup>☆</sup>

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Received 14 February 2006; received in revised form 20 April 2006; accepted 28 April 2006

Available online 16 May 2006

## Abstract

Glutathione *S*-transferases (GSTs) are enzymes that act in excretion of physiologic and xenobiotic substances, protecting cells against chemical toxicity and stress. In this work, we characterized the enzymatic activity of GST in eggs and larvae of cattle tick *Boophilus microplus*, on different days after oviposition and eclosion. The results showed that the GST activity varied depending on the time elapsed after oviposition and eclosion. Molecules involved in mechanism of protection from oxidative stress are correlated with the increase in GST activity. The oxygen consumption kinetics showed a positive correlation with the increase in GST activity during embryogenesis. A high content of thiobarbituric acid reactive substances were observed in egg and larva extracts, indicating that ticks face high oxidative stress during embryogenesis and aging. In eggs and larvae, GST activity can be correlated to kinetic parameters of oxidative stress such as catalase and glutathione. In addition, GST activity showed strong positive correlation with lipid peroxidation, an indication that it plays a role in oxidant defences in eggs.

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**Keywords:** *Boophilus microplus*; Embryonic development; Catalase; Glutathione *S*-transferases; Larval aging; Lipid peroxidation; Oxidative stress; Total glutathione

## 1. Introduction

Ticks (Arachnida) are blood-sucking arthropods that infest a wide array of species (Sauer et al., 1995), including humans and almost every animal of economic importance, and cause significant losses to livestock production (Bowman et al., 1996). The tick *Boophilus microplus* is one of the most important cattle ectoparasites, and causes great economic losses due to the diseases it transmits, along with bovine weight loss and the related high control costs (Jamroz et al., 2000). Studies about ecology, behaviour and physiology of ticks afford a better understanding of these organisms and therefore become important tools to develop new control methods. Due to the

<sup>☆</sup> This paper is part of a special issue of CBP dedicated to “The Face of Latin American Comparative Biochemistry and Physiology” organized by Marcelo Hermes-Lima (Brazil) and co-edited by Carlos Navas (Brazil), René Beleboni (Brazil), Tania Zenteno-Savín (Mexico) and the editors of CBP. This issue is in honour of Cicero Lima and the late Peter W. Hochachka, teacher, friend and devoted supporter of Latin American science.

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rapid increase in pesticide-resistant tick populations (Davey and George, 1998), the study of tick physiology has gained increasing importance as regards the mechanisms involved in detoxification of toxins (Hemingway, 2000; Kostaropoulos et al., 2001). These mechanisms, in general, not only act against specific toxic molecules, but also help in the maintenance of physiologic homeostasis, avoiding oxidative damage generated by reactive oxygen species (ROS). There are two antioxidant systems: non-enzymatic and enzymatic systems. The non-enzymatic antioxidant system use scavenger molecules as GSH (Sies, 1999),  $\alpha$ -tocopherol (vitamin E) and ascorbic acid (vitamin C) (Dandapat et al., 2003), apart from other smaller molecules; the enzymatic antioxidant system is represented by enzymes such as SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase) (Rikans and Hornbrook, 1997), and GSTs (glutathione S-transferases) (Ketterer et al., 1983).

Glutathione S-transferases form a group of ubiquitous enzymes that catalyze the conjugation between glutathione and several molecules, and play the most important role in the cellular detoxification mechanism of xenobiotic and endogenous compounds (Agianian et al., 2003). There are at least two ubiquitously distributed groups of GSTs, microsomal and cytosolic. A third group of GSTs, structurally distinct from the microsomal and cytosolic GSTs, form the Kappa class and were described in mammalian mitochondria and peroxisomes (Morel et al., 2004; Robinson et al., 2004). This superfamily is subdivided into several classes, such as the five cytosolic mammalian classes Alpha, Mu, Pi, Theta and Sigma (Wideström and Mannervik, 1995); some cytosolic classes of GSTs, such as the Omega (Sheehan et al., 2001) and Zeta (Board et al., 1997) classes are represented in a wide range of species, including mammals (Hayes et al., 2005). In rodents and humans, cytosolic GST isoenzymes within a class typically share >40% identity, and those between classes share <25% identity (Hayes et al., 2005). The Epsilon class is present in insects (Ding et al., 2003), and the Phi and Tau classes are present in plants (Dixon et al., 2002).

The chemical exposure of insects is a classical event that selects pesticide resistance, and has been related with a high GST activity (Ketterman et al., 2001; Wei et al., 2001). It has been suggested that the pesticide may conjugate to glutathione by GST and that the compound obtained may therefore act as a detoxification mechanism in arthropods (Beall et al., 1992; Wei et al., 2001). GSTs also perform other physiological roles such as sequestration and transport of endogenous hydrophobic compounds, which include hormones, steroids, heme, bilirubin, bile acids and their metabolites (Salinas and Wong, 1999). The characterization and molecular cloning of GST of *B. microplus* from larvae (He et al., 1999) and from partially engorged female salivary glands have been reported (Rosa de Lima et al., 2002). In our work we identified a relationship between GST and physiologic stress by measuring the oxygen consumption and lipid peroxidation in eggs and larvae, through the analysis of GSTs enzymatic activity. The data obtained were compared with levels of others known antioxidants, CAT and GSH. Based on the correlation between GST activity and oxidative stress

during larva senescence and starvation, we proposed the involvement of GST in oxidative stress protection.

## 2. Materials and methods

### 2.1. Ticks

Fully engorged females of *Boophilus microplus* (Acarina, Ixodidae) (Porto Alegre isolate) were experimentally maintained in the laboratory at 28 °C and 85% relative humidity, and their parasitic life completed in calves housed in individual pens on slatted floors.

### 2.2. Eggs and larvae

Eggs and larvae were collected every 5 days up to the 20th day of oviposition and egg eclosion, respectively. The materials were stored at -70 °C or in liquid nitrogen.

### 2.3. Extract preparation

Egg and larva extracts (100 mg/mL buffer) were macerated in 100 mM Tris-HCl 5 mM EDTA buffer pH 8.0, centrifuged at 15,000×g for 5 min. Next, the supernatants were strained through a 0.45- $\mu$ m pore size filter to remove fat present in the supernatant after centrifugation. The extracts obtained were incubated in ice bath and then immediately tested for the determination of the enzymatic activity with a specific substrate for GST, or for the determination of catalase activity, lipid peroxidation or GSH content.

### 2.4. Biochemical analysis

The GST activity of the extracts was measured as described by Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma) as substrate. About 90  $\mu$ L of the reaction mixture, consisting of 50 mM CDNB in methanol, 5 mM glutathione in 100 mM Tris-HCl pH 7.5, and 10  $\mu$ L of extract in 100 mM Tris-HCl pH 7.5 were tested in a 96-well plate, according to Da Silva Vaz et al. (2004). Buffer without enzyme was used as negative control. The concentration of the product formed was calculated using the extinction coefficient of 9.6  $\text{mM}^{-1} \text{cm}^{-1}$  for *S*-(2,4-dinitrophenyl) glutathione (Wideström and Mannervik, 1995). The protein concentrations of extracts were measured using the Bradford method (Bradford, 1976) with bovine serum albumin as standard. Each assay was run in duplicate and the results were expressed as the calculated mean and standard deviation of three separate experiments.

Oxygen consumption units were assayed using a Clark-type electrode (YSY, model 5775, Yellow Springs, OH, USA), calibrated to 100% with air-saturated buffer at 28 °C, according to Logullo et al. (2002). Reactions were carried out with 200 mg of eggs or larvae in 2 mL of 20 mM PBS buffer pH 7.4, 0.1 mM EDTA, for 60 min. 1 mM KCN was added to inhibit respiration in control samples. Eggs and larvae at 1, 5, 10, 15 and 20 days of age were tested. Assays were carried out in three independent experiments, each in duplicate.

The TBARS (thiobarbituric acid reactive species) assay was used as an index of lipid peroxidation in egg and larva extracts based on the formation of lipid peroxidation products during an acid-heating reaction as previously described by Draper and Hadley (1990). Briefly, the egg and larva extracts prepared were mixed with trichloroacetic acid 10% (v/v), 1 mM butylated hydroxytoluene (BHT), and thiobarbituric acid 0.67% (v/v), and then heated in a boiling water bath for 15 min. TBARS were determined by absorbance at 535 nm and expressed as malondialdehyde (MDA) equivalents (nmol/mg protein). Assays were carried out in three independent experiments, each in duplicate.

The catalase (CAT) activity assay was determined in egg and larva extracts as described by Aebi (1984). Briefly, 20 µL of egg or larva extracts were added to 80 µL of 10 mM Tris–HCl pH 8.0 and 900 µL of 9 mM H<sub>2</sub>O<sub>2</sub>, to a final volume of 1 mL. CAT activity was determined spectrophotometrically by monitoring the disappearance of H<sub>2</sub>O<sub>2</sub> at 240 nm, using the extinction coefficient of 43.6 M<sup>-1</sup> cm<sup>-1</sup> (Aebi, 1984). The results were expressed as U/mg protein. Assays were carried out in three independent experiments, each in duplicate.

The CAT activity in egg and larva extracts was inhibited using aminotriazole, a specific inhibitor of catalase, adapting the method described by Wagner et al. (2005). Briefly, the egg and larva extracts incubated for 30 min with 3-amino triazole (Sigma), at different concentrations. The catalase activity was based on H<sub>2</sub>O<sub>2</sub> degradation, using its extinction molar coefficient, 43.6 M<sup>-1</sup> cm<sup>-1</sup>. The results were expressed as U/mg protein. Assays were carried out in three independent experiments, each in duplicate.

The glutathione (GSH) determination was carried out as described by Akerboom and Sies (1981). Briefly, an equal volume of 2 M HClO<sub>4</sub>, 4 mM EDTA solution was added to egg and larva extracts, and the precipitated proteins were separated by centrifugation for 5 min at 5000×g. The supernatant was neutralized with 2 M KOH in 0.3 M Mops solution in water, centrifuged and assayed. Total glutathione – the sum of reduced glutathione and oxidized glutathione (GSH and GSSG) was determined in the supernatant by a kinetic assay. In the assay, catalytic amounts of GSH and glutathione reductase caused a continuous reduction in 5,5-dithiobis-2-nitrobenzoic acid (DTNB) by nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). The DTNB reduction rate was measured spectrophotometrically at 412 nm. Total glutathione was expressed as µmol/mg protein. Assays were carried out in three independent experiments, each in duplicate.

## 2.5. Statistical analysis

All data values were expressed as mean±S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed. The *t*-tests were used when comparing data between two groups. Significance was set at *p*<0.05. Regression analyses were also performed to compare the changes of various parameters tested for embryonic development and larval aging (Swingscow, 1996).

## 3. Results

### 3.1. Specific enzymatic activity

A gradual increase in GST activity was observed during embryo development. The highest GST activity was found on day 20 after oviposition (0.19 µmol/min/mg protein). GST activity 1 day after egg eclosion decreased significantly and remained approximately so until day 5. On day 10 an increased level was observed and the highest activity in larva was found on day 20 after eclosion (0.24 µmol/min/mg protein) (Fig. 1). GST activity took place exclusively in embryo and larva tissues, since no activity remained in egg shells (data not shown).

### 3.2. Oxygen consumption

To test the relationship between GST activity and oxidative stress, we determined oxygen consumption (Fig. 2) in egg and larva extracts at the same time point used for GST enzymatic activity determination.

In eggs, oxygen consumption increased according to the time elapsed after oviposition. A gradual increase was observed in eggs upon day 20, when the highest level was observed (23.18 µmol O<sub>2</sub>/min mg egg). In contrast, oxygen consumption in larvae was high on day 1 after egg eclosion (18.33 µmol O<sub>2</sub>/min mg larva) and decreased significantly until the last day tested, in particular in 15- and 20-day-old larvae (8.4 µmol O<sub>2</sub>/min mg larva and 10.2 µmol O<sub>2</sub>/min mg larva).

### 3.3. Catalase activity and inhibition assays

CAT activity was measured in egg and larva extracts at the same time point used for GST enzymatic activity determination (Fig. 3). In 1–5- and 10-day old eggs CAT activity remained at a lower level when compared to 15- and 20-day-old eggs (0.78 U/mg protein and 1.49 U/mg protein, respectively). CAT activity was significantly lower in 1-day-old larvae (0.642 U/mg protein) than in 20-day-old eggs, but remained at higher level on all days after eclosion as compared to 1- to 10-day-old eggs

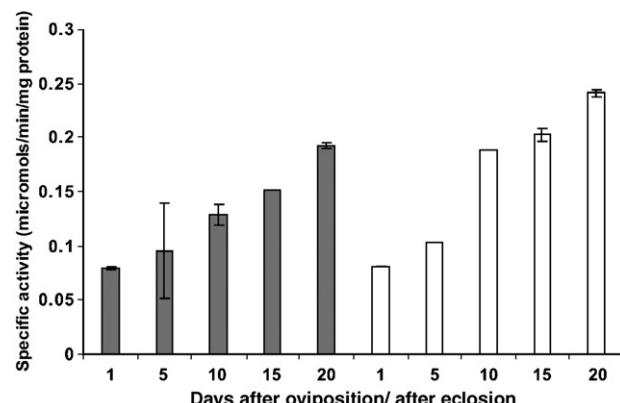


Fig. 1. Enzymatic activity of GST in eggs and larvae of *B. microplus*. Specific enzymatic GST activity in eggs and larvae on different days after oviposition/ eclosion. In grey bars, eggs; in white bars, larvae. The results represent mean±S. D. of three independent experiments, in duplicates.

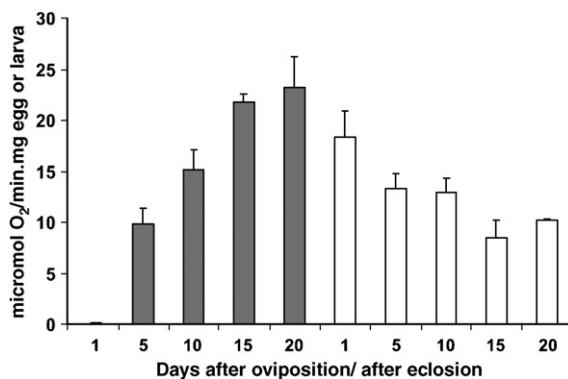


Fig. 2. Oxygen consumption in eggs and larvae. Oxygen consumption was monitored using a Clark-type oxygen electrode. Three assays of 200 mg of eggs or larvae were made in cuvettes containing 2 mL of 20 mM PBS, pH 7.4, 0.1 mM EDTA. In grey bars, eggs; in white bars, larvae. The results represent mean±S.D. of three independent experiments, in duplicates.

(Fig. 3A). The specificity of CAT activity to degrade H<sub>2</sub>O<sub>2</sub> in egg and larva extracts was confirmed by inhibition of the activity by aminotriazole at several concentrations, a compound that is a specific catalase inhibitor (Fig. 3B).

#### 3.4. Glutathione determination

The levels of GSH in eggs increased significantly during embryo development. The highest GST activity was observed in 20-day-old eggs (92.23 μmol/mg protein). A decrease in GSH levels was observed in 1-day-old larvae (2.33 μmol/mg protein) when compared to the days before egg eclosion. Until day 5 after eclosion, GSH concentration was low and gradually increased thereafter until day 20 (Fig. 4).

#### 3.5. Lipid peroxidation

A significant increase in lipid peroxidation in 20-day-old eggs (3.50 nmol MDA/mg protein) was observed when compared to 1-day-old eggs (2.86 nmol MDA/mg protein). In 1-day-old larvae (3.33 nmol MDA/mg protein) and 5-day-old larvae (2.82 nmol MDA/mg protein) the lipid peroxidation was maintained at the same level as in 20-day-old eggs, but decreased significantly in 10-day-old larvae (2.05 nmol MDA/mg protein). Afterwards, a significant increase was observed in 15- and 20-day-old larvae (2.32 and 2.74 nmol MDA/mg protein) in comparison to 10-day-old larvae, but the concentration of MDA produced in 15- and 20-day-old larvae was significantly lower than that observed for 1- and 5-day-old larvae (Fig. 5).

#### 3.6. Correlation between GST activity and other biological parameters

A correlation between GST activity and other biological parameters involved in oxidative stress was established to evaluate the GST role in physiologic oxidative stress. The kinetics of GST activity in eggs and larvae was compared with oxygen consumption, CAT activity and GSH content in its

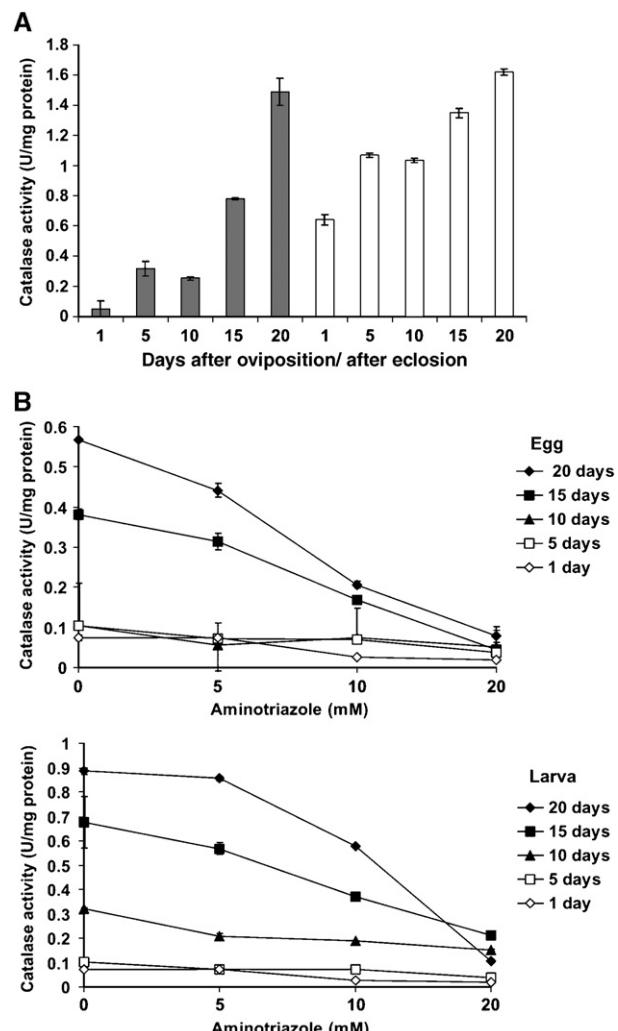


Fig. 3. Catalase activity and inhibition assays. (A) Catalase activity in egg and larva extracts of *B. microplus* in different days. In grey bars, eggs; in white bars, larvae. The results represent mean±S.D. of three independent experiments, in duplicates (B) Catalase inhibition in egg (upper) and larva (lower) extracts of *B. microplus* on different days, using different concentrations of 3-amino triazole (Sigma). The catalase activity was based on H<sub>2</sub>O<sub>2</sub> degradation, using its extinction molar coefficient, 43.6 M<sup>-1</sup> cm<sup>-1</sup>. The results represent mean±S.D. of three independent experiments, in duplicates.

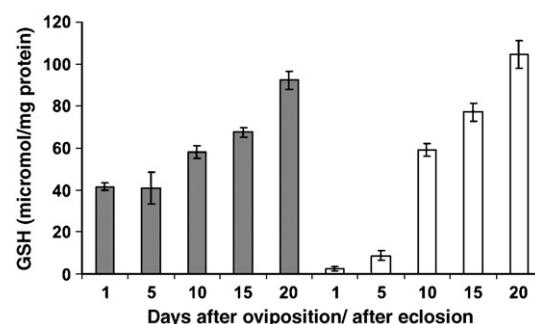


Fig. 4. Determination of total GSH content in egg and larva extracts of *B. microplus*. The GSH content was expressed as μmol/mg protein. In grey bars, eggs; in white bars, larvae. The results represent mean±S.D. of three independent experiments, in duplicates.

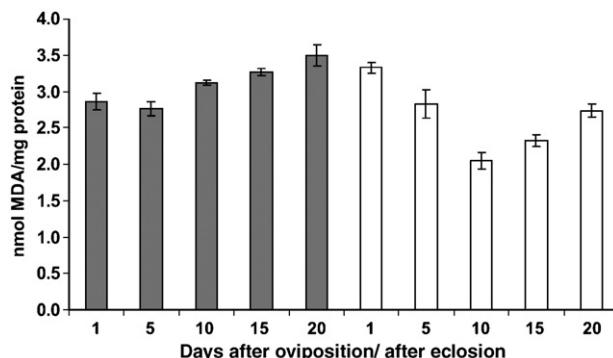


Fig. 5. Determination of thiobarbituric acid reactive substances (TBARS) in eggs and larvae of *B. microplus* on different days. In grey bars, eggs; in white bars, larvae. The results represent mean  $\pm$  S.D. of three independent experiments, in duplicates.

respective life stages. A positive correlation was observed between GST activity and O<sub>2</sub> consumption in embryos ( $r=0.930$ ), and a negative correlation in larvae ( $r=-0.832$ ). There were positive correlations between kinetics of CAT and GST activities in eggs ( $r=0.941$ ) and larvae ( $r=0.879$ ). Also, positive correlations were found between GST activity and GSH content for eggs ( $r=0.985$ ) and larvae ( $r=0.993$ ). The correlation between kinetics of GST activity and lipid peroxidation in eggs was positive ( $r=0.944$ ), whereas in larvae the correlation was negative ( $r=-0.703$ ).

#### 4. Discussion

GSTs are present in almost all eukaryotes and in most of them this happens in multiple isoenzymic forms, constituting a significant intracellular mechanism of detoxification (Papadopoulos et al., 2004). Detoxification is reached through transport and subsequent excretion of toxic organic compounds. Other functions, not associated with detoxification, include repair of macromolecules oxidized by reactive oxygen species, regeneration of S-thiolated proteins, and biosynthesis of physiologically important metabolites (Armstrong, 1997; Sheehan et al., 2001).

In order to understand the role of GST in the tick development, we analyzed its physiological variation during the life stages of eggs and larvae.

The enzymatic GST activity had a significant increase during embryonic development (Fig. 1). The O<sub>2</sub> consumption increases progressively and reached its peak when the embryo is about to hatch (Fig. 2). This increase can be explained by the increased metabolism during the embryonic development in *B. microplus*. We observed strong correlation between O<sub>2</sub> consumption and GST activity.

The enzymatic GST activity increased during the senescence of larvae (Fig. 1). By analyzing the kinetics of GST activity and O<sub>2</sub> consumption during larva aging a strong negative correlation was observed. This decrease in O<sub>2</sub> consumption can be explained by the observation that the larva keeps a high metabolic activity only during the first 5 days after hatching, in order to mature and thus acquire the ability to infest a host. The

optimum period for the Porto Alegre *B. microplus* isolate to infest the bovine is approximately between the 5th and the 7th day post-eclosion (Da Silva Vaz, personal communication). Ogunji and Dipeolu (1977) showed the feeding of larval stages of *Amblyomma variegatum*, *Hyalomma rufipes* and *Boophilus decoloratus* in sheep to be more effective when larvae were fed within 1–4 days of hatching. Also, similar observations were described for other ixodid ticks (Sonenshine, 1991). After this time, the metabolic activity of *B. microplus* larvae decreased because the larva is senescent; however, GST activity remains high, since this enzyme is important to protect against oxidative stress to which the larva is submitted, as shown by the equally high CAT activity and GSH content (Figs. 3 and 4).

One of the consequences of elevated O<sub>2</sub> in the cellular environment is the elevation of ROS levels that could cause oxidative damage, such as nuclear DNA and protein breakdown, as well as lipid peroxidation (Mackay and Bewley, 1989; Barata et al., 2005). The antioxidants CAT and GSH tested presented a positive correlation with GST activity in eggs and in larvae. In fact, a high content of thiobarbituric acid reactive substances was observed in egg and larva extracts, indicating that ticks face high oxidative stress during embryogenesis and aging (Fig. 5). It is possible that the high level of lipid peroxidation verified during embryonic development occurs due to a high concentration of lipids, which are used by the egg as energy source (Logullo et al., 2002; Atella et al., 2005). Since the kinetic parameters of GST and catalase as well as GSH content during embryo development were modulated during different stages, and taking into account the fact that these parameters presented a positive correlation with lipid peroxidation, it is possible to suggest a role of GST in oxidative stress process.

The decreased oxygen consumption during larval aging may be related to metabolic depression and starvation. Phillips et al. (1995) showed that the metabolic rates of *Ornithodoros turicata* adults and larvae were affected by starvation and that oxygen consumption was lower than fed ticks. Some animals maximize their survival time using metabolic depression in response to low concentrations of oxygen available, as a common strategy through which they reduce their metabolism (Lutz and Storey, 1997; Hochachka and Lutz, 2001). Several authors have reported that the level of endogenous antioxidant defences is increased in several species during metabolic depression as a means to protect against oxidative stress. Oliveira et al. (2005) reported an increase in GST activity in gills of *Chasmagnathus granulata* during a metabolic depression induced by anoxia. At low temperatures, the larvae of *B. microplus* (Davey and Cooksey, 1989) and of other ticks (Labruna et al., 2003; Estrada-Pena et al., 2004) reduced their metabolism and managed to survive for long periods without feeding. These data are in accordance with the experiments conducted by Papadopoulos et al. (2004), which demonstrated that adult bees kept at low temperatures and starvation had GST activity increased, suggesting that *B. microplus* larvae decrease metabolism and increase GST activity in order to survive longer in the environment, until a host is found.

The results of this work help to elucidate the role of GST in tick development and assist in the understanding of the importance of GST in the excretion of toxic compounds.

## Acknowledgements

This work was supported by grants from CNPq, PRONEX and FAPERGS.

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